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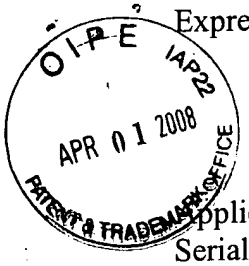

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Antonius Arnoldus Christiaan Jacobs, et al.
Serial No:	10/731,724
Filed:	December 8, 2003
For:	Use of Bacterium for Manufacture of a Vaccine
Confirmation No:	5481
Group Art Unit:	1633
Examiner:	Dr. Sumesh Kaushal
Attorney Ref:	1999.452 US C1

April 1, 2008

APPEAL BRIEF

Mail Stop Appeal
Board of Patent Appeals
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir/Madam:

Pursuant to Appellant's November 1, 2007 Notice of Appeal, Appellant appeals the claim rejections from the June 1, 2007 final Office action that are listed as still pending in the December 11, 2007 advisory action. In support of this appeal, Appellant provides the following information, argument, and fee in accordance with 37 C.F.R. §41.37 and MPEP §§1205 and 1205.02.

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I. REAL PARTY IN INTEREST (37 C.F.R. §41.37(c)(1)(i))

The real party in interest in this appeal is Intervet International B.V. This ownership is evidenced by assignment documents recorded at Reel 014791, Frame 0334 (recorded on June 29, 2004); and Reel 018490, Frame 0365 (recorded on November 8, 2006).

II. RELATED APPEALS AND INTERFERENCES (37 C.F.R. §41.37(c)(1)(ii))

Appellant is not aware of any prior or pending appeal, judicial proceeding, or interference that may be related to, directly affect, or be directly affected by or have bearing on the Board's decision in this appeal.

III. STATUS OF CLAIMS (37 C.F.R. §41.37(c)(1)(iii))

A total of 28 claims have been introduced in this patent application. Claims 1-5 have been canceled. Claims 6-28 remain pending. Every pending claim is rejected. This appeal requests reversal of all the rejections.

IV. STATUS OF AMENDMENTS (37 C.F.R. §41.37(c)(1)(iv))

Appellant filed one amendment after the June 1, 2007 final Office action. That amendment, titled "After Final Amendment F," was filed on November 1, 2007, and amended claims 21 and 28. Based on the December 11, 2007 advisory action, it is Appellant's understanding that the claim amendments have been entered.

V. SUMMARY OF CLAIMED SUBJECT MATTER (37 C.F.R. §41.37(c)(1)(v))

The embodiments in pending claims 6-28 stem from the named inventors' discovery that submucosal administration of live attenuated vaccines generally tends to reduce local adverse reactions that had previously been observed when such vaccines were administered via conventional routes for systemic application (particularly intramuscular administration). This reduction in local reactions is advantageous because it, for example, generally allows for less-attenuated vaccines to be used. *See e.g.*, Appellant's specification, page 1, lines 20-24. This discovery is not limited to any specific live attenuated vaccine. To the contrary, it is generally applicable to *all* live attenuated vaccines, independent of the bacterial strain or method of attenuation. *See, e.g.*, Appellant's specification, page 1, lines 25-28; and Examples 1-3 on pages 7-9.

There are 3 independent claims (*i.e.*, claims 6, 9, and 21). They are summarized as follows:

A. **Claim 6** is directed to a method for administering a live attenuated bacterial vaccine to a mammal. The method comprises injecting an immunogenically effective amount of the vaccine into a submucosal layer of the mammal. Claim 6 is generally supported by Appellant's specification at, for example, page 1, lines 22-28. *See also*, Appellant's specification, page 2, line 30 to page 3, line 23 (defining submucosal administration, and discussing administration sites, depths, and techniques for submucosal administration); page 3, line 25 to page 5, line 29 (discussing example bacteria that are generally suitable for use with the invention); page 6, lines 1-8 (discussing dosage ranges); page 6, lines 9-17 (discussing carrier materials); page 6, lines 18-26 (discussing adjuvants); and Examples 1-3 on pages 7-9 (illustrating and corroborating Appellant's invention with four live bacterial strains and two animal species).

B. **Claim 9** is directed to a method for reducing the amount of adverse reactions in a mammal at an injection site of a live attenuated bacterial vaccine. Here, the live vaccine comprises bacteria that would cause abscess formation if administered intramuscularly. The method comprises administering the vaccine submucosally, whereby the amount of adverse reactions at the injection site is reduced. The reduction is measured by the amount or size of abscesses or lesions at the mucosal

injection site compared to an intramuscular injection site. Claim 9 is generally supported by Appellant's specification at, for example, page 1, lines 20-28; and page 2, lines 4-28. *See also*, Appellant's specification, page 2, line 30 to page 3, line 23 (defining submucosal administration, and discussing administration sites, depths, and techniques for submucosal administration); page 3, line 25 to page 5, line 29 (discussing example bacteria that are generally suitable for use with the invention); page 6, lines 1-8 (discussing dosage ranges); page 6, lines 9-17 (discussing carrier materials); page 6, lines 18-26 (discussing adjuvants); and Examples 1-3 on page 7-9 (illustrating and corroborating Appellant's invention with four live bacterial strains and two animal species).

C. **Claim 21** is directed to a method for systemic application of live attenuated bacteria to a mammal. Here, the live attenuated bacteria would cause abscess and/or lesion formation if administered intramuscularly or intradermally to the mammal. The method comprises administering the live attenuated bacteria submucosally. Any abscess and/or lesion formation at the site of the submucosal administration is less in total size than the abscess and/or lesion formation that would occur if the bacteria were instead administered intramuscularly or intradermally. Claim 21 is generally supported by Appellant's specification at, for example, page 1, lines 20-28; page 2, lines 4-28; and Example 1, page 7, line 1 to page 8, line 20. *See also*, Appellant's specification, page 2, line 30 to page 3, line 23 (defining submucosal administration, and discussing administration sites, depths, and techniques for submucosal administration); page 3, line 25 to page 5, line 29 (discussing example bacteria that are generally suitable for use with the invention); page 6, lines 1-8 (discussing dosage ranges); page 6, lines 9-17 (discussing carrier materials); page 6, lines 18-26 (discussing adjuvants); and Examples 2-3 on page 8, line 21 to page 9, line 26 (further illustrating and corroborating Appellant's invention).

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL (37 C.F.R. §41.37(c)(1)(vi))

Claims 21-28 have been rejected under 35 U.S.C. §112 (first paragraph) for failing to comply with the written description requirement by introducing new matter. Appellant appeals this rejection.

Claims 6-28 have been rejected under the written description requirement of 35 U.S.C. §112 (first paragraph) for failing to show possession of the entire scope of the claimed invention. Appellant appeals this rejection.

Claims 6-28 have been rejected under 35 U.S.C. §112 (first paragraph) for lacking enablement commensurate to the breadth of the claims. Appellant appeals this rejection.

Claims 21-28 have been rejected under 35 U.S.C. §112 (second paragraph) for being indefinite. Appellant appeals this rejection.

VII. ARGUMENT (37 C.F.R. §41.37(c)(1)(vii))

A. *New matter rejection of claims 21-28 under 35 U.S.C. §112 (first paragraph)*

Claims 21-28 have been rejected under 35 U.S.C. §112 (first paragraph) for including new matter. More specifically, the claims have been rejected for reciting a method for "systemic application" of live attenuated bacteria. Appellant requests reversal of this rejection.

This rejection appears to stem from the Examiner's confusion of the terms "mucosal" and "submucosal," as used in Appellant's specification. More specifically, the advisory action states that Appellant's specification excludes systemic application of a vaccine via submucosal administration. As the basis for that contention, the advisory action points to the last two lines of page 1, which state:

> Systemic application comprises all applications in which the vaccine is not applied to the mucosa (mucosal application comprises i.a. oral and intranasal vaccination).

As can be seen, the text excludes *mucosal* administration from the term "systemic application." ***Submucosal* administration, however, is not excluded.** *Submucosal* administration is not the same as *mucosal* administration. Appellant's specification, in fact, draws a clear distinction between the two terms. For example, in lines 3-11 on page 3, Appellant's specification states:

> *Submucosal* application is understood to be administration through the upper layer of the *mucosa*, and into the *submucosa*. The *submucosa* is a well-defined layer, known as such in the art. In principle, there is no limit to depth at which vaccination takes place (i.e. the depth of the tip of the needle), with of course the proviso that vaccination takes place in the *submucosa*. In practice however, the vaccine would not likely be applied deeper than about 5 millimetres from the surface of the *mucosa*. Generally spoken, smaller distances between the *mucosa* and the injection site gives smaller local effects. A very suitable depth would be in the *submucosa* between two and four millimetres below the mucosa. (emphasis added)

And in lines 12-17 on page 3, Appellant's specification further states:

> Another attractive way of application is by using a so-called needle-less injector. The use of these injectors is known from intradermal application, but these injectors are equally suitable for *submucosal* applications. Due to the softness of *mucosal* tissue the vaccine, when applied through a needle-less injector, goes straight through the *mucosa* and will come to a halt in the *submucosal* tissue. (emphasis added).

Simply put, Appellant's specification supports claims directed to a method for systemic application of live attenuated bacteria via submucosal administration. This is clear when Appellant's specification is read in its entirety. For example, on pages 1-3, Appellant's specification expressly states that Appellant's method may be used for systemic application of a live attenuated bacteria:

> **This invention is widely applicable in the field of manufacture of *systemic* vaccines.** It is not restricted to any specific bacterium or a specific disease. **Practically all live attenuated bacteria that are suitable for the manufacture of a live attenuated vaccine for systemic application are equally suitable for use in this specific invention.** Systemic application comprises all applications in which the vaccine is not applied to the mucosa (mucosal application comprises i.a. oral and intranasal vaccination)... Therefore this embodiment of the invention relates to the use of live attenuated bacteria for the manufacture of a vaccine for submucosal administration. Mucosal tissue is found i.a. in the mouth, the nose, the lining of the gut, the eye, the vulva and the lips... Submucosal application is understood to be administration through the upper layer of the mucosa, and into the submucosa. Appellant's specification, page 1, line 25 to page 3, line 4 (emphasis added).

The results in Example 1 on page 7-8 also provide support. That example illustrates the use of submucosal administration of two different live attenuated bacterial strains, and reports systemic protection following vaccination:

> After challenge, the five horses vaccinated submucosally with the TW 928 deletion mutant appeared completely protected. Complete protection was also obtained in the horses vaccinated intramuscularly with the TW 928 deletion mutant.... Therefore it can be concluded that ... full protection can be obtained with suitable vaccine strains regardless the site of administration; intramuscularly or submucosally. Appellant's specification, page 8, lines 12-17.

In view of the foregoing, Appellant respectfully submits that the new matter rejection must be reversed.

B. ***Written description rejection of claims 6-28 under 35 U.S.C. §112 (first paragraph) for failure to show possession of the invention***

Claims 6-28 have been rejected under 35 U.S.C. §112 (first paragraph) for lacking written description showing possession of the invention. Appellant requests reversal of this rejection.

i. *Claim 6*

Claim 6 is directed to a method for administering a live attenuated vaccine to a mammal. The method comprises injecting an immunogenically effective amount of the vaccine into a submucosal layer of the mammal.

In accordance with this invention, the named inventors discovered that submucosal administration of live attenuated vaccines generally tends to reduce local adverse reactions that had previously been observed when such vaccines were administered via conventional routes for systemic application (particularly intramuscular administration). This reduction in local reactions is advantageous because it, for example, generally allows for less-attenuated vaccines to be used. *See e.g.*, Appellant's specification, page 1, lines 20-24. The inventors' discovery is not limited to any specific live attenuated vaccine. To the contrary, **it is generally applicable to *all* live attenuated vaccines, independent of the bacterial strain or method of attenuation.** *See, e.g.*, Appellant's specification, page 1, lines 25-28. This is corroborated by Examples 1-3 in Appellant's specification on pages 7-9, which illustrate the reduction of local reactions by using submucosal administration with **four different live bacterial strains** and **two different animal species**.

Appellant's specification provides direction and guidance for practicing the invention. For example, Appellant's specification provides generally suitable sites for submucosal administration (*see, e.g.*, page 2, line 30 to page 3, line 2 and page 3, lines 18-23); administration depths and techniques (*see, e.g.*, page 3, lines 3-17); dosage ranges (*see, e.g.*, page 6, lines 1-8); suitable carrier materials (*see, e.g.*, page 6, lines 9-17); and suitable adjuvants (*see, e.g.*, page 6, lines 18-26). Appellant's specification also, for example, provides an assortment of example bacteria that are generally suitable for use with the invention. *See, e.g.*, page 3, line 25 to page 5, line 29. And, as noted above, Appellant's specification provides three working examples illustrating submucosal administration with four different live vaccines and two different host species. *See* Examples 1-3, pages 7-9.

The Examiner has rejected claim 6 under 35 U.S.C. §112 (first paragraph) for not having sufficient written description in the specification to support the general applicability of

the recited method to all live attenuated bacterial vaccines. Appellant respectfully requests reversal of this finding.

The law is clear that Appellant's specification must provide a detailed description for only the *novel* aspects of the invention. *See, e.g., Third Wave Tech. v. Stratagene Corp.*, 405 F.Supp.2d 991, 1002 (W.D. Wis. 2005), citing *Genetech v. Novo Nordisk* A/S, 108 F.3d 1361, 1366, 42 USPQ2d 1001, 1005 (Fed. Cir. 1997). *See also, Falkner v. Inglis*, 448, F.3d 1357, 1368, 79 USPQ2d 1001, 1008 (Fed. Cir. 2006) (a requirement that patentees recite known DNA structures would serve no goal of the written description requirement). **In fact, the Federal Circuit discourages inclusion of information that is well-known in the art.** *See, e.g., Hybritech v. Monoclonal Antibodies*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986) (a patent *preferably omits* anything that is well-known in the art). Appellant, therefore, should not be penalized for omitting such information.

In claim 6, the *novel* aspect of Appellant's invention is the submucosal administration. It is undisputed that Appellant's specification provides a sufficient description of this. Claim 6 is instead rejected because it encompasses the use of such administration with any live attenuated bacterial vaccine. Specifically, the Examiner contends that the specification is insufficient to support such breadth because the specification fails to specifically describe how to make live attenuated bacterial vaccines other than *Streptococcus equi*. Appellant respectfully submits that this rejection is contrary to the law.

It is not necessary for Appellant's specification to teach how to attenuate any live bacterial vaccine. **Live attenuated bacterial vaccines, as a general class, were not novel at the time of Appellant's filing. To the contrary, many such vaccines and methods for their preparation were well-known.** To evidence this, the following **Table I** provides a long list of example references discussing live attenuated vaccines and their preparation. These references were located by the Undersigned located during a *non-exhaustive* search. They are dated before or near the time of Appellant's July 29, 1997 priority date, and, therefore, illustrate the plethora of live attenuated bacterial vaccines that existed in the art at the time of Appellant's filing.

Table I
Examples of Live Attenuated Vaccines Known at the Time of Appellant's Filing

Live vaccine	Reference	Filing date (if applicable)	Publication date
Actinobacillus pleuropneumoniae	Inzana, T.J., US Patent 5,429,818, entitled "Non-capsulated mutants of *Actinobacillus pleuropneumoniae* useful as vaccines"	Priority: December 6, 1991 Filed: June 24, 1993	July 4, 1995
Actinobacillus pleuropneumoniae	Segers, R.P.A.M., et al., US Patent 6,013,266, entitled "Live attenuated bacteria of the species *Actinobacillus pleuropneumoniae*"	Priority: April 10, 1997 Filed: April 9, 1998	January 11, 2000
Actinobacillus pleuropneumoniae	Fuller, T.E., et al., US Patent 5,925,354, entitled "Riboflavin mutants as vaccines against *Actinobacillus pleuropneumoniae*"	Priority: November 30, 1995 Filed: October 28, 1996	July 20, 1999
Bordetella bronchiseptica	Switzer, W.P., et al, US Patent 4,225,583, entitled "Intra-respiratory vaccine for prevention of *Bordetella bronchiseptica* infection and method of use"	December 7, 1978	September 30, 1980
Brucella abortus	Adams, L.G., US Patent 5,718,903, entitled "Vaccine comprising *Brucella abortus* which has O polysaccharide antigen absent"	Priority: March 30, 1987 Filed: February 14, 1994	February 17, 1998
Brucella abortus	McEwen, A.D., et al., "Bovine contagious abortion. The use of guinea-pigs in immunisation studies," *The Journal of Comparative Pathology and Therapeutics*, XLIX(2), 97-117 (June 30, 1036)	N/A	June 30, 1936

Live vaccine	**Reference**	**Filing date (if applicable)**	**Publication date**
Clostridium perfringens	Segers, R.P.A.M., et al., US Patent 6,610,300, entitled "*Clostridium perfringens* vaccine"	Priority: June 20, 1997 Filed: June 19, 1998	August 26, 2003
Corynebacterium pseudotuberculosis	Simmons, C.P., "Attenuation and vaccine potential of *aroQ* mutants of *Corynebacterium pseudotuberculosis*," *Infection and Immunity*, 65(8), pp. 3048-3056 (August, 1997)	N/A	August of 1997
Escherichia coli, *Salmonella typhimurium*, & *Shigella flexneri*	Powell, R.J., et al., US Patent 5,997,881, entitled "Method of making non-pyrogenic lipopolysaccharide or A"	Purported priority: November 22, 1995 Filed: February 19, 1997	December 7, 1999
Erysipelothrix rhusiopathiae	Sakano, T., et al., "Effect of attenuated *Erysipelothrix rhusiopathiae* vaccine in pigs infected with porcine reproductive respiratory syndrome virus," *Journal of Veterinary Medical Science*, 59(11), pp. 977-981 (November, 1997)	N/A	November of 1997
Erysipelothrix rhusiopathiae	Sawada, T., et al., "Cross protection of mice and swine inoculated with culture filtrate of attenuated *Erysipelothrix rhusiopathiae* and challenge exposed to strains of various serovars," *American Journal of Veterinary Research*, 48(2), pp. 239-242 (February, 1987)	N/A	February of 1987

Live vaccine	**Reference**	**Filing date (if applicable)**	**Publication date**
Mycobacterium bovis	Flesselles, B., et al., US Patent 6,136,324, entitled "Attenuated strains of mycobacteria"	August 21, 1997	October 24, 2000
Mycobacterium bovis	Barry, III, C.E., et al., US Patent 6,403,100, entitled "Method of attenuating pathogenic mycobacteria and strains of mycobacteria so attenuated"	Priority: July 10, 1997 Filed: July 9, 1998	Appl. published: January 21, 1999 Issued: June 11, 2002
Pasteurella haemolytica	Kucera, C.J., US Patent 4,506,017, entitled "Modified *Pasteurella haemolytica* bacteria"	Priority: April 17, 1981 Filed: January 19, 1983	March 19, 1985
Pasteurella multocida	Maheswaran, S.K., US Patent 3,855,408, entitled "Poultry vaccine"	July 16, 1973	December 17, 1974
Pasteurella multocida	Glisson, J.R., et al., US Patent 4,999,191, entitled "*Pasteurella multocida* Vaccine"	May 5, 1988	March 12, 1991
Rhodococcus equi	Chirino-Trejo, J.M., et al., "Protection of foals against experimental *Rhodococcus equi* pneumonia by oral immunization," *Canadian Journal of Veterinary Research*, 51, pp. 444-447 (1987)	N/A	1987
Salmonella choleraesuis	Smith, H.W., US Patent 3,364,117, entitled "Vaccine for combating *Salmonella choleraesuis* infection"	Priority: September 10, 1963 Filed: September 9, 1964	January 16, 1968
Salmonella dublin & *Salmonella typhimurium*	Stocker, B.A.D., US Patent 4,550,081, entitled "Non-reverting salmonella"	Priority: May 19, 1980 Filed: September 7, 1982	October 29, 1985
Staphylococcus aureus	Australian Patent Appl. AU198285929A1, entitled "Mastitis vaccine"	July 12, 1982	January 20, 1983

Live vaccine	Reference	Filing date (if applicable)	Publication date
Streptococcus pneumoniae	Helms, C.M., "Temperature-sensitive mutants of type I *Streptococcus pneumoniae*: preparation, characterization, and evidence for attenuation and immunogenicity," *The Journal of Infectious Diseases*, 136 (Supp.), pp. S208-S215 (August, 1977)	N/A	August of 1977
Streptococcus suis	Quessy, S., et al., "Immunization of mice against *Streptococcus suis* serotype 2 infections using a live avirulent strain," *Canadian Journal of Veterinary Research (Short Communications)*, 58, pp. 299-301 (1994)	N/A	1994
Streptococcus suis	Busque, P., et al., "Immunization of pigs against *Streptococcus suis* serotype 2 infection using a live avirulent strain," *Canadian Journal of Veterinary Research*, 61, pp. 275-279 (1997)	N/A	1997

The above-cited references are included in the attached **Appendix B** (the Evidence Appendix).

Adding to the abundance of knowledge exemplified in **Table I**, Appellant's specification also specifically identifies a range of example live attenuated bacteria that are generally suitable for use with the invention (*see* page 3, line 25 to page 5, line 29), as well as three working examples illustrating benefits of submucosal administration with four different live vaccines and two different host species (*see* Examples 1-3, pages 7-9).

In view of the foregoing, Appellant respectfully submits that its specification --- when viewed in the context of the art at the time of Appellant's filing --- satisfies the written

description requirement with respect to claim 6. **Such a conclusion accords with the Federal Circuit's holding in *Falkner*:**

> [W]here, as in this case, accessible literature sources clearly provided, as of the relevant date, genes and their nucleotide sequences (here "essential genes"), satisfaction of the written description requirement does not require either the recitation or incorporation by reference (where permitted) of such genes and sequences. *Falkner*, 448, F.3d at 1368, 79 USPQ2d at 1008-1009 (footnote omitted).

As a final note, the Examiner's rejection seems to suggest that attenuation of bacteria requires that the bacteria be usable in a vaccine "without any adverse reaction." *See* June 1, 2007 Office action, page 5, lines 17-20. Appellant respectfully submits that this may stem from a misunderstanding of the term "attenuated" as used in claim 6. The attenuated bacteria recited in claim 6 can have an adverse reaction when used in a vaccine. In fact, such a vaccine *likely will* have an adverse reaction when administered via at least some routes (*e.g.*, intramuscularly). After all, a primary purpose of Appellant's invention is to reduce adverse reactions associated with live attenuated vaccines. This is repeatedly discussed by Appellant's specification. For example, at lines 9-16 on page 2, Appellant's specification states:

> [M]any bacterial IM administered vaccines cause large abscesses at the site of injection. These abscesses may stay there from days to months. In those cases in which a live attenuated bacterium must behave relatively virulent in order to trigger an adequate immune response, the bacterium often replicates at the injection site to such a level that the abscess even bursts. Large intramuscular or skin-abscesses are clearly an unacceptable side-effect of vaccination with bacterial live attenuated strains, but unavoidable if further attenuation spoils the immunogenic potential of the bacterium. This causes the dilemma mentioned above, for which the invention offers a solution.

Moreover, even the live attenuated bacterial vaccine exemplified in Example 1 on page 7-8 of Appellant's specification causes adverse effects, particularly when administered intramuscularly. Simply put, the method of claim 6 can generally be used with any live attenuated vaccine, and is particularly useful with vaccines that cause adverse reactions when administered systemically via a non-submucosal route. Appellant, therefore, respectfully submits that its specification can satisfy the written description requirement without describing how to prepare a live attenuated bacterial vaccine having an absence of adverse reactions.

For at least the reasons discussed above, Appellant respectfully submits that the written description rejection of claim 6 must be reversed.

ii. *Claims 7, 8, and 12-19*

Claims 7, 8, and 12-19 depend directly or indirectly from claim 6. Like claim 6, claims 7, 8, and 12-19 have been rejected for not having sufficient written description in the specification to support the scope of live attenuated bacterial vaccines recited in the claims. Appellant respectfully submits that these claims are supported for at least the same reasons as claim 6.

iii. *Claim 9*

Claim 9 is directed to a method for reducing adverse reactions in a mammal at an injection site of a live attenuated bacterial vaccine. The vaccine comprises bacteria that cause abscess formation when administered intramuscularly. The method comprises administering the vaccine submucosally. The reduction of adverse reactions is measured by the amount or size of abscesses or lesions at the mucosal injection site compared to an intramuscular injection site.

Like claim 6, claim 9 has been rejected for not having sufficient to written description in the specification for live attenuated bacteria other than *Streptococcus equi*. Appellant respectfully submits that claim 9 is supported for at least the same reasons as claim 6.

iv. *Claims 10, 11, and 20*

Claims 10, 11, and 20 depend from claim 9. Like claim 9, claims 10, 11, and 20 have been rejected for not having sufficient written description in the specification to support the scope of live attenuated bacterial vaccines recited in the claims. Appellant respectfully submits that these claims are supported for at least the same reasons as claim 9.

v. *Claim 21*

Claim 21 is directed to a method for systemic application of live attenuated bacteria to a mammal. The bacteria cause abscess and/or lesion formation in the mammal if they are administered intramuscularly or intradermally to the mammal. The method comprises administering the bacteria submucosally to the mammal. Any abscess and/or lesion formation at the site of the submucosal administration is less in total size than the abscess and/or lesion formation that would occur if the bacteria are instead administered intramuscularly or intradermally to the mammal.

Like claim 6, claim 21 has been rejected for not having sufficient written description in the specification for live attenuated bacteria other than *Streptococcus equi*. Appellant respectfully submits that claim 21 is supported for at least the same reasons as claim 6.

vi. *Claims 22-28*

Claims 22-28 depend from claim 21. Like claim 21, claims 22-28 have been rejected for not having sufficient written description in the specification to support the scope of live attenuated bacterial vaccines recited in the claims. Appellant respectfully submits that these claims are supported for at least the same reasons as claim 21.

C. ***Enablement rejection of claims 6-28 under 35 U.S.C. §112 (first paragraph)***

Claims 6-28 have been rejected under 35 U.S.C. §112 (first paragraph) for lacking enablement commensurate to the breadth of the claims. Specifically, the Office action indicates that Appellant's specification is not enabling for live attenuated bacteria other than *Streptococcus equi*. Appellant requests reversal of this rejection.

i. *Claim 6*

Claim 6 is directed to a method for administering a live attenuated vaccine to a mammal. The method comprises injecting an immunogenically effective amount of the vaccine into a submucosal layer of the mammal. As noted above, this method stems from Appellant's discovery that submucosal administration of live attenuated vaccines generally tends to reduce local adverse reactions that had previously been observed when such vaccines

were administered via conventional routes. Appellant's discovery is not limited to any specific live attenuated vaccine. To the contrary, Appellant's discovery is generally applicable to *all* live attenuated vaccines, independent of the bacterial strain or method of attenuation. This is corroborated by Examples 1-3 in Appellant's specification, which illustrate the reduction of local reactions by using submucosal administration with four different live bacterial strains and two different animal species.

The enablement rejection indicates that the specification is insufficient to enable the scope of claim 6 because the specification fails to describe how to make and use live attenuated bacterial strains other than *Streptococcus equi*. Appellant respectfully requests reversal of this finding.

A claim satisfies the enablement requirement if the specification enables a skilled artisan to make and use the claimed invention without "undue experimentation." The necessity for "complex" experimentation does not necessarily equate to "undue" experimentation if those in the art typically engage in such experimentation. *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). *See also,* MPEP §2164.01. Claims are enabled even if "a considerable amount" of experimentation is necessary where the experimentation is "merely routine" or the specification "provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." *In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404. Whether a specification requires undue experimentation depends on multiple factors:

> (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims. *Wands*, 858 F.2d at 737, 8 USPQ2d at 1404.

See also, MPEP §2164.01(a).

Appellant submits that its claims satisfy the enablement requirement for reasons analogous to those in *Wands*. More specifically, in *Wands*, the claims were directed to monoclonal IgM antibodies and an immunoassay using the antibodies. The court found that the claims were enabled even though a skilled artisan practicing the claimed invention would

have to obtain lymphocytes from an immunized animal; fuse the lymphocytes with myeloma cells; and then perform multiple screening steps to identify and separate out hybridomas, hybridomas producing antibodies to the desired antigen, and finally hybridomas producing antibodies having the claimed affinity. *Wands*, 858 F.2d at 739-740, 8 USPQ2d at 1404-1406. In finding enablement, the court noted: (1) the specification provided guidance for practicing the invention, (2) the specification provided working examples, (3) the level of skill in the art was high, (4) the methods needed to practice the invention were well known in the art, and (5) the nature of the technology involved screening to identify antibodies with the desired characteristics. *Wands*, 858 F.2d at 740, 8 USPQ2d at 1406. **Using reasoning analogous to *Wands*, Appellant's claim 6 also should be found to satisfy the enablement requirement. Specifically:**

1. **Appellant's specification provides guidance for practicing the invention.** For example, Appellant's specification provides generally suitable sites for submucosal administration, administration depths and techniques, dosage ranges, suitable carrier materials, and suitable adjuvants. Appellant's specification also identifies a wide range of example live bacteria that are generally suitable for use with the invention.

2. **Appellant's specification provides three working examples illustrating submucosal administration with four different live vaccines and two different host species.** These examples include two different live attenuated bacterial strains.

3. **The skill level in the art and nature of the technology are analogous to those in *Wands*.**

4. **The methods needed to practice the invention are well known in the art.** As to live attenuated vaccines in particular, there was an extensive understanding in the art relating to methods for making and generally using live vaccines at the time Appellant's application was filed. The scientific literature from the time of Appellant's filing is replete with discussions relating to the development and use of live attenuated vaccines. The non-exhaustive list of references cited in the above **Table I** demonstrates this.

A finding of enablement is further supported by the Federal Circuit's holding in *Falkner*. In that case, the court found that the Board did not err in finding enablement for a poxvirus vaccine claim, even in the absence of a poxvirus working example, where there was

a working example for a herpesvirus vaccine. *Falkner*, 448 F.3d at 1365, 79 USPQ2d at 1006. In justifying its holding, the court acknowledged that "great expenditures of time and effort were ordinary in the field of vaccine preparation." *Id.*

Simply put, Appellant's specification --- particularly when viewed in the context of the knowledge in the art at the time of Appellant's filing --- enables claim 6. Accordingly, Appellant respectfully submits that the enablement rejection of claim 6 must be reversed.

ii. *Claims 7, 8, and 12-19*

Claims 7, 8, and 12-19 depend directly or indirectly from claim 6. Like claim 6, claims 7, 8, and 12-19 have been rejected for not having sufficient description in the specification to enable the scope of live attenuated bacterial vaccines recited in the claims. Appellant respectfully submits that these claims are enabled for at least the same reasons as claim 6.

iii. *Claim 9*

Claim 9 is directed to a method for reducing the amount of adverse reactions in a mammal at an injection site of a live attenuated bacterial vaccine. The vaccine comprises bacteria that cause abscess formation when administered intramuscularly. The method comprises administering the vaccine submucosally. The reduction of adverse reactions is measured by the amount or size of abscesses or lesions at the mucosal injection site compared to an intramuscular injection site.

Like claim 6, claim 9 has been rejected for not enabling the use of live attenuated bacteria other than *Streptococcus equi*. Appellant respectfully submits that claim 9 is enabled for at least the same reasons as claim 6.

iv. *Claims 10, 11, and 20*

Claims 10, 11, and 20 depend from claim 9. Like claim 9, claims 10, 11, and 20 have been rejected for not having sufficient description in the specification to enable the scope of live attenuated bacterial vaccines recited in the claims. Appellant respectfully submits that these claims are enabled for at least the same reasons as claim 9.

v. *Claim 21*

Claim 21 is directed to a method for systemic application of live attenuated bacteria to a mammal. The bacteria cause abscess and/or lesion formation in the mammal if they are administered intramuscularly or intradermally to the mammal. The method comprises administering the bacteria submucosally to the mammal. Any abscess and/or lesion formation at the site of the submucosal administration is less in total size than the abscess and/or lesion formation that would occur if the bacteria are instead administered intramuscularly or intradermally to the mammal.

Like claim 6, claim 21 has been rejected for not enabling the use of live attenuated bacteria other than *Streptococcus equi*. Appellant respectfully submits that claim 21 is enabled for at least the same reasons as claim 6.

vi. *Claims 22-28*

Claims 22-28 depend from claim 21. Like claim 21, claims 22-28 have been rejected for not having sufficient description in the specification to enable the scope of live attenuated bacterial vaccines recited in the claims. Appellant respectfully submits that these claims are enabled for at least the same reasons as claim 21.

D. ***Indefiniteness rejection of claims 21-28 under 35 U.S.C. §112 (second paragraph)***

Claims 21-28 have been rejected under 35 U.S.C. §112 (second paragraph) for being indefinite. Specifically, the Examiner states that the claims are indefinite because it is unclear how a "systemic application" can be achieved using submucosal administration. The Examiner contends that systemic application cannot exist when a vaccine is administered at a specific site, particularly when the route of administration is intramuscular, intradermal, or submucosal. Appellant requests reversal of this rejection.

Appellant respectfully submits that this rejection is without merit. It is well-known that "systemic application" encompasses any administration that has a systemic effect (*i.e.*, affecting the body generally) rather than simply a local effect. It is also well-known that such an effect can be achieved using an extravascular route of administration. *See, e.g., The Merck*

Veterinary Manual, p. 1517 (7th Ed. Merck & Co. Rahway, N.J., 1991) (noting that intramuscular injections are commonly used to achieve systemic immunity). A systemic effect may occur when, for example, antigens from the vaccine are absorbed from the site of administration into the bloodstream and create an immune response elsewhere. Alternatively (or in addition), antigens from the vaccine may cause an immune response at the site of administration, which, in turn, produces antibodies that end up in the bloodstream to create a systemic effect.

Appellant's specification expressly states that the claimed submucosal route of administration is useful for systemic application of a vaccine. *See, e.g.*, Appellant's Specification, page 1, lines 25-26. And this is corroborated by Example 1 on pages 7-8 of Appellant's specification. Specifically, Example 1 demonstrates that the submucosal administration caused a systemic immune response in horses that provided full protection against a challenge. Thus, Appellant's specification has provided a written description of the claimed subject matter, illustrated the use of the claimed subject matter, and shown that the claimed subject matter works. **The second paragraph of 35 U.S.C. §112 does not require Appellant to explain why the invention works.** *Fromson v. Advance Offset Plate, Inc.*, 720 F.2d 1565, 1570 (Fed. Cir. 1983) ("it is axiomatic that an inventor need not comprehend the scientific principles on which the practical effectiveness of his invention rests..."). *See also*, *Diamond Rubber Co. of New York v. Consolidated Rubber*, 220 U.S. 428, 435-36, 31 S.Ct. 444, 447 (1911) ("It is certainly not necessary that [the inventor] understand or be able to state the scientific principles underlying his invention."). Thus, Appellant respectfully submits that the rejection must be reversed.

VIII. DESCRIPTION OF CLAIMS APPENDIX (37 C.F.R. §41.37(c)(1)(viii))

An appendix containing a copy of all the claims involved in the appeal is attached.

IX. DESCRIPTION OF EVIDENCE APPENDIX (37 C.F.R. §41.37(c)(1)(ix))

An appendix containing a copy of each reference listed in **Table I** (on pages 14-17 above) is attached. Appellant submitted the references in support of arguments that Appellant made in its June 22, 2006 Amendment D. Appellant also cited the references in support of arguments that Appellant made in its November 1, 2007 After Final Amendment F. The references are as follows:

1. Inzana, T.J., US Patent 5,429,818, entitled "Non-capsulated mutants of *Actinobacillus pleuropneumoniae* useful as vaccines" (issued July 4, 1995).

2. Segers, R.P.A.M., et al., US Patent 6,013,266, entitled "Live attenuated bacteria of the species *Actinobacillus pleuropneumoniae*" (issued January 11, 2000).

3. Fuller, T.E., et al., US Patent 5,925,354, entitled "Riboflavin mutants as vaccines against *Actinobacillus pleuropneumoniae*" (issued July 20, 1999).

4. Switzer, W.P., et al, US Patent 4,225,583, entitled "Intra-respiratory vaccine for prevention of *Bordetella bronchiseptica* infection and method of use" (issued September 30, 1980).

5. Adams, L.G., US Patent 5,718,903, entitled "Vaccine comprising *Brucella abortus* which has O polysaccharide antigen absent" (issued February 17, 1998).

6. McEwen, A.D., et al., "Bovine contagious abortion. The use of guinea-pigs in immunisation studies," *The Journal of Comparative Pathology and Therapeutics*, XLIX(2), 97-117 (June 30, 1036).

7. Segers, R.P.A.M., et al., US Patent 6,610,300, entitled "*Clostridium perfringens* vaccine" (issued August 26, 2003).

8. Simmons, C.P., "Attenuation and vaccine potential of *aroQ* mutants of *Corynebacterium pseudotuberculosis*," *Infection and Immunity*, 65(8), pp. 3048-3056 (August, 1997).

9. Powell, R.J., et al., US Patent 5,997,881, entitled "Method of making non-pyrogenic lipopolysaccharide or A" (issued December 7, 1999).

10. Sakano, T., et al., "Effect of attenuated *Erysipelothrix rhusiopathiae* vaccine in pigs infected with porcine reproductive respiratory syndrome virus," *Journal of Veterinary Medical Science*, 59(11), pp. 977-981 (November, 1997).

11. Sawada, T., et al., "Cross protection of mice and swine inoculated with culture filtrate of attenuated *Erysipelothrix rhusiopathiae* and challenge exposed to strains of various serovars," *American Journal of Veterinary Research*, 48(2), pp. 239-242 (February, 1987).

12. Flesselles, B., et al., US Patent 6,136,324, entitled "Attenuated strains of mycobacteria" (issued October 24, 2000).

13. Barry, III, C.E., et al., US Patent 6,403,100, entitled "Method of attenuating pathogenic mycobacteria and strains of mycobacteria so attenuated" (application published January 21, 1999; issued June 11, 2002).

14. Kucera, C.J., US Patent 4,506,017, entitled "Modified *Pasteurella haemolytica* bacteria" (issued March 19, 1985).

15. Maheswaran, S.K., US Patent 3,855,408, entitled "Poultry vaccine" (issued December 17, 1974).

16. Glisson, J.R., et al., US Patent 4,999,191, entitled "*Pasteurella multocida* Vaccine" (issued March 12, 1991).

17. Chirino-Trejo, J.M., et al., "Protection of foals against experimental *Rhodococcus equi* pneumonia by oral immunization," *Canadian Journal of Veterinary Research*, 51, pp. 444-447 (1987).

18. Smith, H.W., US Patent 3,364,117, entitled "Vaccine for combating *Salmonella choleraesuis* infection" (issued January 16, 1968)

19. Stocker, B.A.D., US Patent 4,550,081, entitled "Non-reverting salmonella" (issued October 29, 1985).

20. Australian Patent Appl. AU198285929A1, entitled "Mastitis vaccine" (published January 20, 1983).

21. Helms, C.M., "Temperature-sensitive mutants of type I *Streptococcus pneumoniae*: preparation, characterization, and evidence for attenuation and immunogenicity," *The Journal of Infectious Diseases*, 136 (Supp.), pp. S208-S215 (August, 1977).

22. Quessy, S., et al., "Immunization of mice against *Streptococcus suis* serotype 2 infections using a live avirulent strain," *Canadian Journal of Veterinary Research (Short Communications)*, 58, pp. 299-301 (1994).

23. Busque, P., et al., "Immunization of pigs against *Streptococcus suis* serotype 2 infection using a live avirulent strain," *Canadian Journal of Veterinary Research*, 61, pp. 275-279 (1997).

X. DESCRIPTION OF RELATED PROCEEDINGS APPENDIX (37 C.F.R. §41.37(c)(1)(x))

None.

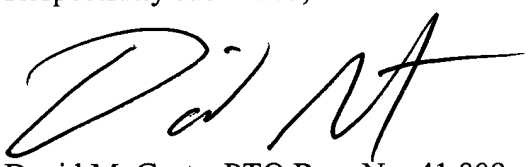
XI. Fee payment and extension request

Appellant authorizes the Commissioner to charge Deposit Account No. **02-2334** for the $510.00 fee under 37 CFR §41.20(b)(2) for filing this appeal. Appellant also requests a three-month extension to file this brief, and authorizes the Commissioner to charge Deposit Account No. **02-2334** for the corresponding extension fee under 37 CFR §1.17(a)(5). Appellant does not believe that any other fee is due in connection with this filing. If, however, Appellant does owe any such fee(s), the Commissioner is hereby authorized to charge the fee(s) to Deposit Account No. **02-2334**. In addition, if there is ever any other fee deficiency or overpayment in connection with this patent application, the Commissioner is hereby authorized to charge such deficiency or overpayment to Deposit Account No. **02-2334**.

* * * * * * * * *

Appellant submits that the pending claims are in condition for allowance, and requests the rejections in the June 1, 2007 final Office action (to the extent still pending according to the December 11, 2007 advisory action) be reversed, and this application be allowed.

Respectfully submitted,



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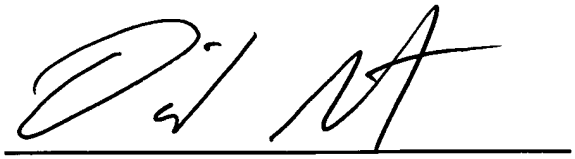

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enclosures

APPENDIX A
Claims Appendix (37 C.F.R. §41.37(c)(1)(viii))

6. A method for administering a live attenuated bacterial vaccine to a mammal, wherein the method comprises injecting into a submucosal layer of the mammal an immunogenically effective amount of the vaccine.

7. The method according to claim 6, wherein the live attenuated bacterium of the vaccine is selected from the group consisting of *Actinobacillus equili, Actinobacillus pleuropneumoniae, Actinomyces pyogenes, Bordetella bronchiseptica, Brucella abortus, Clostridium perfringens, Corynebacterium bovis, Corynebacterium pseudotuberculosis, Erysipelothrix rhusiopathiae, Escherichia coli, Haemophilus parasuis, Leptospira canicola, Leptospira hardjo, Leptospira icterohaemorrhagiae, Leptospira pomona, Mycobacterium bovis, Mycoplasma bovis, Mycoplasma hyopneumoniae, Noccardia asteroides, Pasteurella haemolytica, Pasteurella multocida, Pseudomonas mallei, Rhodococcus equi, Salmonella cholerasuis, Salmonella dublin, Salmonella typhimurium, Serpulina hyodysenteriae, Staphylococcus aureus, Streptococcus agalactiae, Streptococcus equi, Streptococcus pneumoniae, Streptococcus suis, Streptococcus uberis,* and *Streptococcus zooepidemicus*.

8. The method according to claim 6, wherein the mammal is a horse.

9. A method for reducing the amount of adverse reactions in a mammal at an injection site of a live attenuated bacterial vaccine, wherein:

the method comprises administering submucosally the vaccine, whereby the amount of adverse reactions at the injection site is reduced,

the live bacterial vaccine comprises bacteria that cause abscess formation when administered intramuscularly, and

the reduction of the amount of adverse reactions is measured by the amount or size of abscesses or lesions at the mucosal injection site compared to an intramuscular injection site.

10. The method according to claim 9, wherein the vaccine is administered into the submucosa of the labiae.

11. The method according to claim 9, wherein the live attenuated bacterium of the vaccine is selected from the group consisting of *Actinobacillus equili, Actinobacillus pleuropneumoniae, Actinomyces pyogenes, Bordetella bronchiseptica, Brucella abortus, Clostridium perfringens, Corynebacterium bovis, Corynebacterium pseudotuberculosis, Erysipelothrix rhusiopathiae, Escherichia coli, Haemophilus parasuis, Leptospira canicola, Leptospira hardjo, Leptospira icterohaemorrhagiae, Leptospira pomona, Mycobacterium bovis, Mycoplasma bovis, Mycoplasma hyopneumoniae, Noccardia asteroides, Pasteurella haemolytica, Pasteurella multocida, Pseudomonas mallei, Rhodococcus equi, Salmonella cholerasuis, Salmonella dublin, Salmonella typhimurium, Serpulina hyodysenteriae, Staphylococcus aureus, Streptococcus agalactiae, Streptococcus equi, Streptococcus pneumoniae, Streptococcus suis, Streptococcus uberis,* and *Streptococcus zooepidemicus.*

12. The method according to claim 6, wherein the live attenuated bacterium of the vaccine is selected from the group consisting of *Actinobacillus pleuropneumoniae, Bordetella bronchiseptica, Brucella abortus, Clostridium perfringens, Corynebacterium pseudotuberculosis, Erysipelothrix rhusiopathiae, Escherichia coli, Mycobacterium bovis, Mycoplasma hyopneumoniae, Pasteurella haemolytica, Pasteurella multocida, Rhodococcus equi, Salmonella cholerasuis, Salmonella dublin, Salmonella typhimurium, Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus suis,* and *Streptococcus uberis.*

13. The method according to claim 12, wherein the mammal is a horse.

14. The method according to claim 12, wherein the mammal is a ruminant.

15. The method according to claim 12, wherein the mammal is a pig.

16. The method according to claim 12, wherein the mammal is a dog.

17. The method according to claim 6, wherein the mammal is a ruminant.

18. The method according to claim 6, wherein the mammal is a pig.

19. The method according to claim 6, wherein the mammal is a dog.

20. The method according to claim 9, wherein the live attenuated bacterium of the vaccine is selected from the group consisting of *Actinobacillus pleuropneumoniae, Bordetella bronchiseptica, Brucella abortus, Clostridium perfringens, Corynebacterium pseudotuberculosis, Erysipelothrix rhusiopathiae, Escherichia coli, Mycobacterium bovis, Mycoplasma hyopneumoniae, Pasteurella haemolytica, Pasteurella multocida, Rhodococcus equi, Salmonella cholerasuis, Salmonella dublin, Salmonella typhimurium, Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus suis,* and *Streptococcus uberis*.

21. A method for systemic application of live attenuated bacteria to a mammal, wherein:

the method comprises administering the live attenuated bacteria submucosally to the mammal,

the live attenuated bacteria cause abscess and/or lesion formation in the mammal if the live attenuated bacteria are instead administered intramuscularly or intradermally to the mammal, and

any abscess and/or lesion formation at the site of the submucosal administration is less in total size than the abscess and/or lesion formation that would occur if the bacteria are instead administered intramuscularly or intradermally to the mammal.

22. A method according to claim 21, wherein the live attenuated bacteria cause abscess and/or lesion formation in the mammal if the live attenuated bacteria are administered intramuscularly to the mammal.

23. The method according to claim 21, wherein the live attenuated bacteria are selected from the group consisting of *Actinobacillus equili, Actinobacillus pleuropneumoniae, Actinomyces pyogenes, Bordetella bronchiseptica, Brucella abortus, Clostridium perfringens, Corynebacterium bovis, Corynebacterium pseudotuberculosis, Erysipelothrix rhusiopathiae, Escherichia coli, Haemophilus parasuis, Leptospira canicola, Leptospira hardjo, Leptospira icterohaemorrhagiae, Leptospira pomona, Mycobacterium bovis, Mycoplasma bovis, Mycoplasma hyopneumoniae, Noccardia asteroides, Pasteurella haemolytica, Pasteurella multocida, Pseudomonas mallei, Rhodococcus equi, Salmonella cholerasuis, Salmonella dublin, Salmonella typhimurium, Serpulina hyodysenteriae, Staphylococcus aureus, Streptococcus agalactiae, Streptococcus equi, Streptococcus pneumoniae, Streptococcus suis, Streptococcus uberis,* and *Streptococcus zooepidemicus*.

24. The method according to claim 21, wherein the live attenuated bacteria are selected from the group consisting of *Actinobacillus pleuropneumoniae, Bordetella bronchiseptica, Brucella abortus, Clostridium perfringens, Corynebacterium pseudotuberculosis, Erysipelothrix rhusiopathiae, Escherichia coli, Mycobacterium bovis, Mycoplasma hyopneumoniae, Pasteurella haemolytica, Pasteurella multocida, Rhodococcus equi, Salmonella cholerasuis, Salmonella dublin, Salmonella typhimurium, Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus suis,* and *Streptococcus uberis*.

25. The method according to claim 21, wherein the mammal is a horse.

26. The method according to claim 21, wherein the mammal is a ruminant.

27. The method according to claim 21, wherein the mammal is a pig.

28. The method according to claim 21, wherein the mammal is a dog.

APPENDIX B
Evidence Appendix (37 C.F.R. §41.37(c)(1)(ix))

The following are copies of references cited in Table I on pages 14-17.

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BOVINE CONTAGIOUS ABORTION.

THE USE OF GUINEA-PIGS IN IMMUNISATION STUDIES.

By A. D. McEWEN and R. S. ROBERTS,

South-Eastern Agricultural College, Wye, Kent.

THE few records of immunisation experiments against *Br. abortus* infection in guinea-pigs are insufficient to warrant any conclusions regarding the value of these animals for this type of work. Experiments with killed cultures have been recorded by Ascoli (1916), Stafseth (1920), Hagan (1922) and Gwatkins (1931). Ascoli and Stafseth concluded that no immunity was produced, but their methods of testing immunity were most probably too severe. Hagan paid adequate attention to experimental details but the numbers of animals used were small, only seven vaccinated animals being available for comparison with seven controls; he considered, however, that *Br. abortus* infection developed more slowly in the vaccinated animals. Gwatkins used repeated large inoculations of killed cultures but no immunity was demonstrated.

More significant results are reported from the inoculation of living cultures of low virulence. Huddleson (1924) stated that a living culture of a non-virulent strain of *Br. abortus* had been found to protect guinea-pigs from abortion, when exposed to infection by feeding the animals with virulent micro-organisms, but no details of his experiments are given. Schroeder and Cotton (1925) reported upon the inoculation of guinea-pigs with a strain which only caused lesions when very large doses were given. The strain, however, was recoverable from the spleens of guinea-pigs two months after their inoculation with moderate doses. They vaccinated guinea-pigs with heavy suspensions of this living culture and exposed some of these animals and control guinea-pigs to a subcutaneous infection with a bovine strain of *Br. abortus*; the other vaccinated animals, together with controls, were infected with a porcine strain of *Br. abortus*. The quantities of these

respective test inoculations are not given, but, on the whole, when the animals were killed and examined the vaccinated guinea-pigs showed fewer lesions or less extensive lesions than the controls. No bacteriological examinations were made.

Were it possible to increase the resistance of both guinea-pigs and cattle by the use of the same vaccine, the guinea-pig might prove a most valuable laboratory animal for the routine examination of vaccines used in the field.

The primary objective of the experiments now to be summarised was to ascertain whether a strain of *Br. abortus* of low virulence stimulated a measurable resistance to a subsequent exposure to an infection with a virulent strain of *Br. abortus*. Only one strain of low virulence has been tested, Strain No. 45. This strain was isolated some years ago in this country from material of bovine origin, most probably prior to 1922, but the exact date of its isolation is uncertain. It behaves as a typical bovine strain; it is agglutinated well by positive sera, and throughout the course of these experiments it has remained smooth, being heat-stable according to the criteria adopted by Pandit and Wilson (1932). Comparative tests have shown that the strain grows equally as well in ordinary atmospheric air as in an atmosphere containing 10 per cent. CO_2 gas, irrespective of whether the seed material is derived from a stock culture or from the tissues of an inoculated animal.

In 1931 a few preliminary experiments demonstrated the low virulence of the strain for guinea-pigs and suggested the possibility of its possessing antigenic value, and in September 1931 the current sub-culture of that date was arbitrarily designated Sub-culture X1, and sub-cultures from that time have been numbered in series.

Since March 1930, Strain 45 has been maintained exclusively on liver agar slants. The virulent strains used in testing the resistance or immunity of guinea-pigs have been grown exclusively on the same medium. All suspensions of the organisms used in the preparation of vaccine and for the test inoculations, etc., have been prepared from 48-hour growths on liver agar, washed off with normal saline solution and standardised to a required opacity, using Brown's opacity tubes for this purpose.

On a table supplied with the opacity tubes, the numbers of organisms in suspensions of *Br. melitensis* of opacity comparable to those of the different tubes in the set are given. It has been arbitrarily assumed that suspensions of *Br. abortus* contain a like number of micro-organisms to comparable suspensions of *Br. melitensis*. On this basis and accepting the numbers of *Br. melitensis* given in the above-mentioned table as correct, so in the succeeding records the numbers of *Br. abortus* in the different suspensions used are given, the reference to the actual opacity of the suspensions generally being omitted. Suspensions for the

agglutination test have been prepared either from Strain 45 or from another smooth strain. The same suspension has always been used throughout any one experiment.

In order to ensure a CO_2 sensitivity in the strain used to test for immunity it has been necessary to use more than one CO_2 sensitive strain throughout the experiments. In one experiment vaccinated animals were deliberately infected with a virulent ærobic strain.

Prior to 1933, when the experimental guinea-pigs were killed and examined, the spleen was removed and portions of the organ thoroughly teased with needles; a loopful of spleen tissue was then inoculated on to agar slants. When other tissues were examined for evidence of infection these were treated in a similar manner. From 1933 onwards the spleens were weighed and afterwards reduced to a pulp by scraping with a spatula; a quantity of the pulp was reserved for the inoculation of agar slants and the remainder of the pulp transferred to sterile thick-walled test tubes containing a number of glass beads, a measured volume of sterile saline solution was then added, the tube closed with a rubber stopper and vigorously shaken. A 1 : 10 dilution of the spleen pulp suspension was made in a second tube. From these two suspensions, amounts of 0·2 c.c. were sown on fuchsin agar plates, or on agar slants in large test tubes of 1 in. internal diameter and from the number of colonies which formed the number which theoretically could have been obtained from the whole spleen was estimated. From comparisons made on cultures from a large number of guinea-pigs, the enumeration of colonies obtained from spleen emulsions provided no more reliable data than that obtained by the direct inoculation of spleen pulp on agar.

When material from animals vaccinated with living cultures of Strain 45 was examined the absence of growth in atmospheric air was regarded as evidence of the elimination of the vaccine strain from the body.

Attempts have been made to passage Strain 45 through guinea-pigs. It was found impossible to passage the strain directly from animal to animal by the inoculation of spleen tissue, the strain invariably being lost in the second passage animal. The strain, however, was generally recoverable from the spleens of guinea-pigs inoculated seven to ten days previously with large numbers of bacteria, but growth was not abundant, only a few colonies being obtained per slant richly seeded with spleen pulp; subcultivation of these primary growths yielded rich cultures which were in turn inoculated into guinea-pigs, and so on. From February to May 1935, the strain was passaged through a series of six guinea-pigs by such means.

When Strain 45 was tested for pathogenicity in 1931 it was found that the subcutaneous or intraperitoneal inoculation of guinea-pigs each with 768 million micro-organisms or the

subcutaneous inoculation of twice that number failed to produce any macroscopic evidence of infection or to produce agglutinins in titres higher than 1 : 160. However, from the spleens of some animals a few colonies of *Br. abortus* were obtained even up to 33 days after inoculation. By June of 1934 such *in vivo* survival powers as the strain may have possessed three years previously had probably diminished, because the micro-organism was not recoverable after the fourth week in any one instance from the spleens of guinea-pigs inoculated subcutaneously with the relatively enormous dose of 61,480 million micro-organisms. The titre of the serum remained low, even after these large inoculations. In one experiment where 20 guinea-pigs were used, the serum from one animal agglutinated at a titre of 1 : 640 and the serum from another at 1 : 320, but the remaining sera all had lower titres.

The vaccination experiments of 1931 further indicated the absence of invasive properties of the strain even at that time, no evidence having been obtained from the examination of guinea-pigs in immunisation experiments of the persistence of the strain in the body of the guinea-pigs a few weeks after inoculation.

The conclusion that the strain was of low virulence for the guinea-pigs throughout the four years under consideration is justifiable. Furthermore, from the protocols of the last vaccination experiment which are summarised later, it is apparent that even after serial passage through six guinea-pigs the virulence of the passaged strain had not been exalted to an appreciable extent, if at all.

Immunisation Experiments.

1931-32 *Experiments.*

Preliminary experiments made in 1931 indicated that guinea-pigs inoculated once with 1,537 million living micro-organisms of Strain 45 possessed greater resistance to infection caused by applying a drop of a suspension of a recently isolated CO_2 sensitive strain of *Br. abortus* containing 2,305 million organisms per cubic centimetre, to the eye, than control animals. Furthermore, the inoculation of three doses of living micro-organisms in ascending magnitude at approximately weekly intervals, stimulated an appreciable resistance in a group of guinea-pigs which included a number of female animals; and a comparison of pregnant vaccinated animals with pregnant control animals showed a greater susceptibility to uterine infection and abortion among the latter. The experiments of that year, however, indicated that when a number of guinea-pigs were inoculated with the same living vaccine and divided into two groups and later infected in a comparable manner but on different dates, the apparent resistance to infection of the groups might differ.

An opportunity to carry out further experiments did not occur until September 1932, when eleven male guinea-pigs each received

three subcutaneous inoculations, each consisting of 1,537 million organisms of Strain 45 Sub-culture X5. Fifty-nine days after receiving the final inoculation the vaccinated animals and an equal number of control male guinea-pigs received subcutaneously a test inoculation of a virulent CO_2 sensitive strain of *Br. abortus*. The test inoculation consisted of 1 c.c. of a suspension of organisms equivalent in opacity to No. 1 tube of the Brown scale (containing 768 million organisms per cubic centimetre) diluted 10^7. The dilutions, however, were all made with the same pipette, and therefore the approximate number of organisms inoculated cannot be even roughly estimated. Equal numbers of the vaccinated and control animals were killed and examined later at intervals of 43, 57, 60 and 71 days respectively. None of the vaccinated animals showed lesions, whereas the characteristic splenic enlargement of a *Br. abortus* infection was shown by all the controls, and two of these animals, one killed on the forty-third day and the other on the sixtieth day, each showed abscessation of one testicle. *Br. abortus* was recovered in culture from the spleen of one vaccinated guinea-pig but was not obtained from the livers of any of the animals. *Br. abortus* was, on the other hand, recovered in culture from the spleens of all the control animals and from the livers of four of them. The titres of the sera when the animals were killed further reflected the decided resistance of the vaccinated animals to infection. The titres of vaccinated animals were 1 : 40, 1 : 80, 1 : 80, 1 : 40, 0, 1 : 80, 1 : 320, 0, 1 : 160, 0 and 1 : 80, and those of the control animals 1 : 640, 1 : 2,560, 1 : 2,560, 1 : 1,280, 1 : 10,240, 1 : 2,560, 1 : 640, 1 : 640, 1 : 80, 1 : 640 and 1 : 2,560.*

The results of this experiment were unequivocally in favour of the vaccinated group which showed, according to the criteria of the examination, a complete resistance in 90 per cent. of the animals to an exposure which caused infection in 100 per cent of the controls.

1933—*Experiment on Male Guinea-pigs.*

The next experiment was commenced in May 1933 and it was designed in the hope of repeating the favourable results of the preceding experiment: and also to try to compare the immunising properties of vaccines consisting of living organisms of Strain 45 Sub-culture X226, obtained by repeated sub-cultivation at intervals of one to two days; of heat killed suspensions of Strain 45 Sub-culture X9; and of formalin sterilised suspensions of the same strain and sub-culture, with suspensions of live organisms of the same.

Male guinea-pigs were used and vaccinated three times at intervals of 14 days. The animals given the living suspensions of *Br. abortus* received 3,074 million organisms at each inoculation;

* 0 = No agglutination at 1 : 20.

those receiving suspensions sterilised by heat and formalin respectively, 30,740 million organisms at each inoculation. The sterilisation of the former was carried out by heating in the water bath at 65° C. for one hour; the sterilisation by formalin was accomplished by adding sufficient formalin to the suspension of the organisms to give a final concentration of 0·5 per cent. formalin and incubating at 37° C. for 48 hours.

The vaccinated guinea-pigs and control animals each received a test inoculation 14 days after the administration of the last inoculation of vaccine. The test inoculation was similar to that employed in the preceding experiment of September 1932, except in so far as the sixth instead of the fourth sub-culture of the CO_2 sensitive strain was used. The guinea-pigs were killed and examined 31, 34, 35, 39, 40, 45, 48 and 49 days after infection, as far as possible equal numbers being killed from each group at the same time.

The results of this experiment were not very satisfactory and may be summarised as follows: Of 15 animals inoculated with live vaccine from Sub-culture X9, eleven, or 73 per cent., were infected, and one, or 6 per cent., showed macroscopic lesions; of 13 animals inoculated with live vaccine from Sub-culture X226, ten, or 77 per cent., were found to be infected, and two, or 15 per cent., showed macroscopic lesions; of 15 animals inoculated with heat killed vaccine, fourteen, or 93 per cent., were infected, and eight, or 53 per cent., showed macroscopic lesions; of 15 inoculated with formalinised vaccine, twelve, or 80 per cent., were infected, and four, or 26 per cent., showed macroscopic lesions; 16 out of 16 controls were infected, and six, or 37 per cent., showed macroscopic lesions.

The experiment, though of limited value, does not suggest that the repeated sub-cultivation had depleted Strain 45 of antigenic properties and it indicates that heat killed vaccine may be of little value.

In a further experiment in 1933 comparisons were made between male guinea-pigs inoculated four times with 61,840 million live organisms at intervals of one week and similar animals inoculated in the same manner but with a number of micro-organisms ten times smaller. The animals were infected six weeks after the last inoculation of vaccine. Owing to intercurrent disease the numbers of the animals in experiment were considerably reduced but the summarised results in Table I show that vaccination with either the large or the smaller dose had increased the animals' resistance to infection.

1934—*Experiments on Male Guinea-pigs.*

An experiment was commenced in May 1934, and was designed to test the effect of large multiple doses of vaccine; multiple smaller sized doses; a single large dose and single smaller dose;

TABLE I.

No. of G. Pigs per group.	*No. and Percentage found infected.*	*No. and Percentage showing Macroscopic Lesions.*	*Average No. of Colonies from Spleen Cultures, Infected Animals only considered.*	*No. and Percentage with infected Livers.*	*Average No. of Colonies from Liver Cultures.*	*Average Titre of Serum.*

TABLE I.

	No. of G. Pigs per group.	*No. and Percentage found infected.*	*No. and Percentage showing Macroscopic Lesions.*	*Average No. of Colonies from Spleen Cultures, Infected Animals only considered.*	*No. and Percentage with infected Livers.*	*Average No. of Colonies from Liver Cultures.*	*Average Titre of Serum.*
Group 1. Large multiple inoculations	10	2 20 per cent.	0 0 per cent.	7	0 0 per cent.	0	25
Group 2. Moderate multiple inoculations	6	2 33 per cent.	0 0 per cent.	8	0 0 per cent.	0	38
Group 5. Controls ...	8	8 100 per cent.	8 100 per cent.	104	3 37 per cent.	3	640

In estimating the average titre of the serum, the dilution of the serum at the end point of agglutination was regarded as a fraction.

TABLE II.

PLAN OF EXPERIMENT OF MAY, 1934.

Group of Guinea Pigs.	*Weeks.* 1st.	2nd.	3rd.	4th.	5th.	6th.	7th.	8th.	9th.	10th.	15th.	20th.	25th.
1 ...	10uk.	10uk.	10uk.	10uk.						Inf.	Ex.		
2 ...	1ul.	1ul.	1ul.	1ul.						"	"		
3 ...	10ul.	10ul.	10ul.	10ul.						"	"		
4 ...				1ul.						"	"		
5 ...				0·1ul.						"	"		
6 ...						1ul.	1ul.	1ul.	1ul.	"	"		
7 ...						10ul.	10ul.	10ul.	10ul.	"	"		
8 ...									1ul.	"	"		
9 ...									10ul.	"	"		
10 ...										"	"		
11 ...		1ul.	1ul.	1ul.	1ul.							Inf.	Ex.
12 ...		10ul.	10ul.	10ul.	10ul.							"	"
13 ...												"	"

10uk. = 10 units of killed vaccine, one unit containing 6,148 million micro-organisms.
1ul. = 1 unit of living vaccine, one unit containing 6,148 million micro-organisms.
10ul. = 10 units of living vaccine, one unit containing 6,148 million micro-organisms.
Inf. = Infected with 23,000 organisms of a virulent CO_2 sensitive strain of *Br. abortus*. In preparing these and all subsequent test inoculations a separate pipette was used in making each dilution.
Ex. = Killed and examined.

TABLE III.

Group and Vaccine.	Dose of Vaccine.	Time in Weeks before Infection.	No. of G. Pigs per Group.	No. and Percentage Infected.	No. and Percentage Heavily Infected in Spleen.	No. and Percentage Infected in Liver.	Average Weights of Spleens.	No. of Sera with a Titre of 1 : 10 and Higher and Average Titre of these Sera.
1. Heat killed	L.M.	6	15	12	6	1	1·06	14
				80 per cent.	40 per cent.	6·6 per cent.		56
2. Living	S.M.	6	17	1	0	0	0·77	16
				5·9 per cent.	0 per cent.	0 per cent.		32
3. Living	L.M.	6	16	4	0	0	0·84	13
				25 per cent.	0 per cent.	0 per cent.		29
4. Living	S.S.	6	18	3	0	0	1·00	14
				16·6 per cent.	0 per cent.	0 per cent.		34
5. Living	L.S.	6	18	3	0	0	0·85	17
				16·6 per cent.	0 per cent.	0 per cent.		41
6. Living	S.M.	1	18	18	2	1	0·97	18
				100 per cent.	11 per cent.	5·5 per cent.		134
7. Living	L.M.	1	17	16	1	1	0·89	16
				88·2 per cent.	5·8 per cent.	5·8 per cent.		117
8. Living	S.S.	1	17	14	1	1	0·89	16
				82·3 per cent.	5·8 per cent.	5·8 per cent.		50
9. Living	L.S.	1	17	12	3	3	0·93	17
				70·5 per cent.	17·4 per cent.	17·4 per cent.		45
10. Controls	—	—	18	18	8	7	1·36	18
				100 per cent.	44 per cent.	38·5 per cent.		147
11. Living	S.M.	15	18	17	2	3	1·45	18
				94 per cent.	11·5 per cent.	16·5 per cent.		241
12. Living	L.M.	15	16	13	2	2	1·11	16
				71·1 per cent.	12·4 per cent.	2·4 per cent.		106
13. Controls	—	—	18	18	6	8	1·88	18
				100 per cent.	33·3 per cent.	44 per cent.		461

Aerobic strains of *Br. abortus* were isolated from five animals in each of Groups 6 and 7 and from 6 animals in each of Groups 8 and 9 indicating that the vaccine strain had not been eliminated from the body. Ordinarily the vaccine strain is not recoverable from the tissues 6 weeks after vaccination, and its isolation from the animals in these groups must be attributed to the effect of inoculating the animals with a virulent culture shortly after they had been vaccinated.

L.M. = large multiple : S.M. = small multiple : L.S. = large single : S.S. = small single.

and also to test the immunity at different periods of time after the last inoculation of vaccine. Furthermore, it was planned to test a vaccine composed of dead organisms sterilised by heat. The living vaccine was in each case prepared from Strain 45 Sub-culture X14; the dead vaccine was prepared from the same strain Sub-culture X13. The vaccine sterilised by heat had been maintained at a temperature of 60° C. for one hour, no preservative was added, and the vaccine was kept in the ice chest.

The plan of the experiment and its results are given in Tables II and III.

From the above summary it is evident that where the living vaccine was used satisfactory results were obtained with either multiple or single inoculations of large or smaller doses of vaccine when the test inoculation was given six weeks after the last inoculation of vaccine, the best results being shown by Groups 2, 3, 4 and 5. When the results of these groups are considered together they show that out of 69 guinea-pigs 58, or 84 per cent., completely resisted an infection which was sufficient to infect all of the 18 control animals.

When the test inoculation was given one week after the inoculation of the last dose of vaccine the results were not satisfactory, but in none of these vaccinated groups is there any evidence that the inoculation, even of large quantities of the vaccine strain only one week before infection, had increased the susceptibility to infection. Indeed, the evidence indicates some slight increase in resistance, as nine out of 69 guinea-pigs in Groups 6, 7, 8 and 9 resisted infection. Furthermore, in some of the remaining 60 guinea-pigs it is probable that the infection which was found was due to a persistence of the vaccine strain.

In this experiment those animals which received one inoculation only of vaccine, Groups 8 and 9, and could have developed no immunity from previous inoculations of vaccine, were significantly no more susceptible than the controls, Group 10, although viable organisms from the vaccine must have been present in the bodies of the vaccinated animals when the test inoculation was administered.

In that portion of the experiment where the resistance of the animals was tested 15 weeks after the last inoculation of vaccine the results are interesting. Although the animals in Groups 11 and 12 were but very little more resistant than the controls, they had been inoculated with comparable and in some instances with exactly the same vaccine which had produced a very definite immunity in the majority of the guinea-pigs in Groups 2, 3, 4 and 5. Therefore the failure to demonstrate a definitely increased resistance in the animals in Groups 11 and 12 cannot be attributed to any variation in the vaccine. Groups 11 and 12 were infected 15 weeks after vaccination, whereas Groups 2, 3, 4 and 5 were exposed to infection only six weeks after vaccination, but it is

most unlikely that a detectable diminution in immunity could occur between the seventh and the sixteenth week following vaccination. Moreover, in a later experiment immunity was shown to persist for at least 19 weeks. In the present experiment the same virulent strain of *Br. abortus* was used in the preparation of the test inoculations for all the groups of guinea-pigs, and when test inoculations were prepared comparable methods were employed in standardising the suspensions of culture to a like capacity and in the preparation of the requisite dilutions. Nevertheless it is probable that the inconstant results of this and of some other experiments were due to a variation in the severity of the test inoculations used and that these variations were uncontrollable by the methods employed.

The object of the next experiment was to test the effect of exposing vaccinated guinea-pigs to an infection with a virulent ærobic strain of *Br. abortus* and later exposing the animals to a second infection, but with a CO_2 sensitive strain of *Br. abortus*. Furthermore, the experiment was so arranged that it might furnish some data upon the duration of increased resistance in guinea-pigs.

Three groups of vaccinated and three groups of control guinea-pigs were used. The vaccinated groups are designated V.1, V.2 and V.3, and the control groups C.1, C.2 and C.3. All the animals in the vaccinated groups were inoculated at the same time and with the same vaccine, each animal receiving one inoculation of 6,148 million live organisms of Strain 45, Sub-culture X13.

Eight weeks after vaccination the animals in Groups V.1 and V.2, and the control guinea-pigs in Group C.1, were each inoculated with 30,700 organisms of a virulent ærobic strain of *Br. abortus*. Nineteen weeks after receiving this test inoculation the animals in Groups V.1 and C.1 were killed and examined. *Br. abortus* was isolated from three of the vaccinated animals, in two instances in very small numbers only, and from eight of the ten control guinea-pigs, thereby indicating that the vaccinated animals had possessed an increased resistance to infection. The guinea-pigs in Groups V.3 and C.3 were each inoculated with 30,700 organisms of a CO_2 sensitive strain 19 weeks after the animals in Group V.3 had been vaccinated. Seven weeks later the animals in both of these groups were killed and examined, that is on the twenty-sixth week of the experiment. Of the nine surviving vaccinated animals none was found infected with *Br. abortus*, whereas this micro-organism was recovered from eight out of ten animals in the control group. This portion of the experiment therefore furnished evidence that an immunity produced by vaccination was present 19 weeks after vaccination. The vaccinated animals in Group V.2 which had been inoculated eight weeks after vaccination with the virulent ærobic strain of *Br. abortus* were each inoculated 18 weeks later, that is 26 weeks after vaccina-

tion, with 30,700 organisms of the CO_2 sensitive strain previously used for the inoculation of Groups V.3 and C.3; the control animals in Group C.2 were similarly inoculated at this time. Six weeks later the animals in Groups V.2 and C.2 were killed and examined. *Br. abortus* was recovered from three of the nine surviving animals in Group V.2 and in each instance the recorded organisms were ærobic. All of the eight surviving control animals in Group C.2 showed a heavy infection with a CO_2 sensitive strain of *Br. abortus*.

In considering the results obtained in the six groups it appears that in Groups V.1 and C.1 the vaccination had produced a partial immunity to the inoculation of the ærobic strain of *Br. abortus* eight weeks later, and in Groups V.3 and C.3 a definite immunity to the inoculation of the CO_2 sensitive strain 19 weeks after vaccination. Furthermore, that vaccinated guinea-pigs exposed to infection with the ærobic strain, Group V.2, were able to withstand a later exposure to a CO_2 sensitive strain although certain of the guinea-pigs were at the time actually infected. The resistance of these guinea-pigs to the superimposition of infection is of particular interest and the experiment indicates the feasibility of testing the immunity produced by exposure to ærobic strains of some degree of pathogenicity, either in previously vaccinated animals or in normal animals, when the final test for increased resistance is carried out with a CO_2 sensitive strain. Finally, the experiment showed that an immunity response to the inoculation of the non-virulent Strain 45 persisted for a period of at least 19 weeks.

A summary of the experiment is given in Table IV.

TABLE IV.

Groups of G. Pigs.	*Weeks of Experiment.*				*No. of G. Pigs Examined.*	*Percentage found Infected.*
	8th.	*19th.*	*26th.*	*32nd.*		
Vaccinated 1	Inf. with ærobic	Examine			10	30 with ærobic.
Control 1	„	„			10	80 with ærobic.
Vaccinated 2	„		Inf. with CO_2	Examine	9	33 with ærobic only.
Control 2			„	„	8	100 with CO_2.
Vaccinated 3		Inf. with CO_2	Examine		9	None.
Control 3		„	„		10	80 with CO_2.

1934—*Experiments on Female Guinea-pigs.*

It had been noted during 1931 that infected pregnant female guinea-pigs showed a heavy *Br. abortus* infection of the uterus; and that vaccinated pregnant guinea-pigs after exposure to infection, though sometimes infected in the spleen, might be free from

a uterine infection, or less severely infected in that organ than comparable control animals. If by vaccination with a non-virulent strain of *Br. abortus* it were possible to increase the resistance of the pregnant uterus to infection, even although the immunity were insufficient to afford the guinea-pig complete protection against infection, it would encourage the hope that the comparable treatment of the bovine might give equally favourable results in the protection of the pregnant uterus.

In the experiment about to be described female guinea-pigs were used and were infected after mating. Although abortion in infected guinea-pigs has been observed to occur with considerable regularity, nevertheless it was difficult to detect in individuals kept in groups, therefore reliance had to be placed on evidence of uterine infection at *post-mortem* examination, and the guinea-pigs were killed when it was considered most likely to find the majority of the animals pregnant or to have but recently aborted or given birth to young. Infection of the pregnant uterus was demonstrated by inoculating portions of the placentæ on to liver agar slopes. In cases of uterine infection this seed material affords luxuriant growths of *Br. abortus*, the organisms, indeed, may frequently be demonstrated in large numbers in suitably stained smears made from the tissues at the apposition of the fœtal and maternal discs. Following the abortion, or possibly the parturition, of an infected guinea-pig, single or multiple abscessation, with honeycombing and thickening of the uterine wall in the neighbourhood of the abscessation, frequently occurs. These lesions have been observed exclusively in *Br. abortus* infected animals, and in many cases the thickish cream-coloured pus from the abscesses yields abundant and pure cultures of *Br. abortus*, but at other times media inoculated with material from the uterine lesions is overgrown by contaminating bacteria.

Two groups of virgin female guinea-pigs were vaccinated, the one, Group (*a*), on March 21st, 1934; and the other, Group (*b*), on June 11th, 1934. The animals in both groups were each inoculated with 61,470 million organisms of Strain 45, those inoculated into Group (*a*) being from Sub-culture X15 and those used for the inoculation of Group (*b*) from Sub-culture X17. On June 11th a suitable number of male animals were introduced into each vaccinated group and also into a control group of normal females, Group (*c*). On July 2nd, 1934, all the female guinea-pigs received one drop on the eye of a saline suspension of a CO_2 sensitive strain of *Br. abortus* containing approximately 2,000 million bacteria per cubic centimetre. These animals were again infected in the same manner by applying a similar suspension to the opposite eye on July 5th. This double test dose of a virulent organism was very severe, experiments having demonstrated that very dilute suspensions of *Br. abortus*, when instilled on to the eye may set up infection with regularity. The animals

were killed from the twenty-eighth to the thirty-sixth day after first being infected, there being twelve females in Group (*a*), eleven in Group (*b*) and 21 in the control group (*c*). Unfortunately bacteriological examinations were made on only ten animals from Group (*a*), nine from Group (*b*) and 18 from the control group (*c*).

These bacteriological examinations showed that all the guinea-pigs were infected but the different groups were not affected to a like degree, the disease being mildest in Group (*a*) and most severe among the controls. Thus the average spleen weights were: Group (*a*) 1·72 grammes, Group (*b*) 2·27 grammes, and Group (*c*) 3·06 grammes, and the average estimated number of colonies per spleen was: Group (*a*) 9,600, Group (*b*) 61,800, and Group (*c*) 331,000. The superiority of the animals in Group (*a*) was maintained in the bacteriological examination of the livers and uteri. The examinations of the former organ showed none infected from Group (*a*); while four out of nine from Group (*b*) and 14 out of 18 in Group (*c*) were infected. The examinations of the uteri were particularly interesting and are summarised in Table V.

TABLE V.

Condition of Uterus.			*Cultures made from the Uterus or Placentae.*		
	Groups.			*Groups.*	
A.	*B.*	*C.*	*A.*	*B.*	*C.*
P.	P.	P.	———	None made	———
P.	P.	Abt.		" "	
		P.		" "	
P.	Abs.	P.	Neg.	Contin.	Contin.
P.	P.	P.	"	Neg.	"
P.		P.	Contin.		"
P.		P.	Neg.		"
		Not P.N.			Neg.
		"			"
P.	Abs.	"	Neg.	Contin.	"
		P.			Contin.
		P.			"
Abs.	Abs.	Abs.	O.B.	O.B.	O.B.
P.	Abs.	P.	Neg.	O.B.	Contin.
Not P.N.	P.	E.P.	"	Neg.	"
P.	P.	Abs.	"	Contin.	"
E.P.	P.	Abs.	"	"	"
	Not P.N.	Not P.N.		Neg.	Neg.
		"			"
		E.P.			"
		Abs.			Contin.

P. = Pregnant; E.P. = Early pregnancy; Abt. = Abortion; Abs. = Abscessation of uterus; Not P.N. = Not pregnant, uterus normal.

Neg. = No bacterial growth; Contin. = Continuous growth of *Br. abortus* on surface of agar; O.B. = No growth of *Br. abortus* but a growth of other bacteria.

Considering those animals examined bacteriologically, it is seen that seven animals in Group (*a*) were well advanced in

pregnancy, and in only one of these was the uterus found infected. One animal in Group (*a*) showed abscessation of the uterus and one was in an early stage of pregnancy. *Br. abortus* was not isolated from the uterus of either of these animals but the abscessation is regarded as the direct sequel to *Br. abortus* infection. Therefore of the guinea-pigs which were pregnant or showed evidence of having been pregnant, only two out of nine, or 22 per cent., were infected or had suffered from a uterine infection with *Br. abortus*.

Seven control guinea-pigs were well advanced in pregnancy and all were infected in the uterus; four were suffering from abscessation of the uterus; two were in early pregnancy and one of these was infected. Therefore, out of 13 guinea-pigs which were pregnant or showed evidence of having been pregnant twelve, or 92 per cent., were, or had been, infected in the uterus with *Br. abortus*.

The animals in Group (*b*) were little, if at all, more resistant to uterine infection than the controls. The unsatisfactory results shown by Group (*b*) might be attributable to the use of a deteriorated vaccine. The only difference between the vaccines was that the vaccine inoculated into Group (*b*) animals was prepared from a culture two sub-cultures removed from that which provided the vaccine for Group (*a*), but it is unlikely that this was responsible for a deterioration of the vaccine.

The experiment in general confirmed the impression formed in 1931 that a considerable resistance to uterine infection could be induced by immunisation.

The next experiment was commenced in November 1934, its chief purpose being to obtain further information regarding the resistance of vaccinated guinea-pigs to *Br. abortus* infection acquired during pregnancy; both living and formalinised vaccines were tested.

Forty-eight female guinea-pigs believed to be non-pregnant were selected and divided into six groups. These animals were kept under observation for eight weeks prior to any inoculations being made and at the end of that period used for experiment. The groups were then treated as follows:—

Group 1.—Inoculated subcutaneously once with 6,148 million live organisms of Strain 45, Sub-culture X14.

Group 2 (six guinea-pigs).—Inoculated subcutaneously with 30 million live organisms, Strain 45, Sub-culture X14.

Group 3.—Inoculated subcutaneously with 30,740 million dead organisms, Strain 45, Sub-culture X14, formalinised vaccine.

Group 4.—Inoculated subcutaneously with 30 million dead organisms, Strain 45, Sub-culture X14, formalinised vaccine.

Group 5.—Inoculated subcutaneously with 30,740 million dead organisms of a virulent CO_2 sensitive strain No. 253, formalinised vaccine, prepared from Sub-culture 10.

Group 6.—Controls.

Four weeks after vaccination two male guinea-pigs were placed with each group of females and two weeks later the females each received a subcutaneous inoculation of what was estimated to be 30,700 live abortion bacteria of Strain 253, Sub-culture 12; the guinea-pigs were killed and examined 44 and 58 days later. The results of these examinations are shown in Table VI.

TABLE VI.

Group 1.	Live vaccine, moderate dose.	
	Titres of sera after vaccination and before infection :	1 : 80–1 : 320.
	No. of guinea-pigs examined, 8.	
	" " found pregnant, 6.	
	" " infected in the uterus, 0.	
Group 2.	Live vaccine, small dose.	
	Titres of sera after vaccination and before infection :	Negative at 1 : 10–1 : 160.
	No. of guinea-pigs examined, 6.	
	" " found pregnant, 1.	
	" " infected in the uterus, 1.	
Group 3.	Live vaccine strain sterilised by formalin.	
	Titres of sera after vaccination and before infection :	Negative at 1 : 10–1 : 40.
	No. of guinea-pigs examined, 8.	
	" " found pregnant, 1 (dead fœtus).	
	" " infected in the uterus, 6.	
Group 4.	Live vaccine strain sterilised by formalin, small dose.	
	Titre of sera after vaccination and before infection :	All negative at 1 : 10.
	No. of guinea-pigs examined, 8.	
	" " found pregnant, 0.	
	" " infected in the uterus, 6.	
Group 5.	Virulent strain sterilised by formalin.	
	Titres of sera after vaccination and before infection :	Negative at 1 : 10–1 : 40.
	No. of guinea-pigs examined, 8.	
	" " found pregnant, 0.	
	" " infected in the uterus, 5.	
Group 6.	Controls.	
	No. of guinea-pigs examined, 8.	
	" " found pregnant, 0.	
	" " infected in the uterus, 4.	

The control guinea-pigs, Group 6, were all heavily infected in the spleen and in all cases *Br. abortus* was recovered from the liver. None of the animals was pregnant at the time of the examination and in four cases macroscopic examination of the uterus showed no evidence of the animals having been pregnant. From the appearance of the uteri of the four remaining animals it is probable that they had aborted. *Br. abortus* was isolated in culture from the uteri of four of the eight guinea-pigs and the uteri of two of these four animals showed no macroscopic evidence of abortion having occurred. It is, however, probable that these two latter animals had aborted as *Br. abortus* is rarely recovered, by the technique used, from the uteri of infected non-pregnant guinea-pigs unless it be soon after abortion or from uteri showing

abscessation. The combined macroscopic and bacteriological evidence therefore indicates that six of the guinea-pigs had had infected uteri and probably had aborted.

In Group 1, vaccinated with a moderately large number of living micro-organisms of Strain 45, well-developed fœtuses were found in six of the guinea-pigs. With one of the two remaining animals it was very doubtful whether abortion had occurred, but in the other there was no evidence that the animal had been pregnant. In no case was *Br. abortus* isolated from the uterus or the placentæ, showing that the animals possessed a relatively high degree of resistance to a uterine infection. It has been found that *Br. abortus* is recoverable with much greater regularity from the placentæ of infected guinea-pigs than from the uterine mucosa of non-pregnant animals, which reflects more forcefully the immunity of the uterus in the six vaccinated pregnant animals. As a group, and as individuals, the infection of the spleen was much lighter than in the controls; indeed, two of the eight vaccinated animals were found free from infection altogether and in no instance was *Br. abortus* isolated from the liver tissue.

It is difficult to decide whether the resistance of the animals in Group 2 had been increased. Five of the animals were not pregnant and in three of these there was no macroscopic evidence of the uterus having been gravid, and *Br. abortus* was not recovered from the uteri; probably both of the others had aborted and *Br. abortus* was isolated from the uterus of one of them. The sixth guinea-pig was pregnant but its uterus was not infected. The spleens and livers showed a degree of infection comparable to that shown by the controls. The guinea-pigs do not compare favourably with those in Group 1, indicating that a single small inoculation of vaccine was relatively unsatisfactory.

In the remaining groups 3, 4 and 5 there was evidence that the majority of the animals had aborted and there was no evidence of any increased resistance to infection. It may therefore be concluded that the formalinised vaccines whether prepared from Strain 45 or from the virulent Strain 253, and this strain was used to test immunity, were of little value. Even when the formalinised vaccines were inoculated in very large quantity they were definitely very inferior to the vaccine composed of living non-virulent micro-organisms.

In conformity with what has been the general experience throughout all the experiments, the agglutination titres at the time the guinea-pigs were killed were highest among the control animals and those groups which showed no increased resistance to infection.

Attention should be directed to the pre-infection agglutination titres of the sera from some of the groups. The inoculation of very large numbers of killed micro-organisms of the virulent strain in no case produced a titre higher than 1 : 40. The inoculation of

similar numbers of killed micro-organisms of the non-virulent Strain 45 produced very similar titres, 1 : 40 again being the highest recorded. The inoculation of a relatively small number, 30 million, sterilised organisms of Strain 45 failed to produce agglutinins at all; however, 30 million of the live organisms of this strain caused the appearance of agglutinins in the majority of the animals, a titre of 1 : 160 being reached in one instance. Those animals in Group 1 inoculated with the largest number of living non-virulent organisms produced titres distinctly higher than the animals receiving five times as many killed organisms of the same strain or of the virulent strain. In all of the groups except No. 1 the titres showed a very marked rise after exposure to a small number of virulent micro-organisms. A general consideration of these results suggests : (1) That following sterilisation by formalin, Strain 45 stimulated the production of agglutinins as effectively as did the sterilised virulent strain; (2) that when the living organisms were inoculated the higher titres produced by the virulent strain depended upon the invasiveness of the strain and its multiplication and persistence for some considerable time in the tissues; (3) and that the relatively feeble production of agglutinins following the inoculation of living non-virulent micro-organisms was due to their inability to persist in the body tissues.

1935—*Experiments on Male Guinea-pigs.*

An experiment was carried out in which groups of guinea-pigs were vaccinated at different times and all infected at the same time. The chief object of the experiment was to test the length of time immunity lasted. Unfortunately no satisfactory immunity was demonstrated in any of the vaccinated groups although the methods of vaccination and of infecting the animals were regarded as comparable to those used in other experiments where a definitely increased resistance had been shown. By this time four years had elapsed since the earlier experiments had indicated that the inoculation of guinea-pigs with living organisms of Strain 45 stimulated an increased resistance to a subsequent *Br. abortus* infection. The poor results obtained in the last experiment suggested the possibility of a decided antigenic deterioration of the vaccine strain. Accordingly, arrangements were now made to compare the immunity of guinea-pigs inoculated with vaccine prepared from the ordinary sub-culture of Strain 45, with the immunity of guinea-pigs inoculated with vaccine prepared from a culture which had been passaged intermittently through a short series of guinea-pigs. In anticipation of an experiment of this nature the intermittent passage of Strain 45 had commenced in February 1935, and in June 1935 a sub-culture was available from Strain 45 which had been passaged through a series of six guinea-pigs and vaccine was prepared from it; the vaccine itself was obtained from the third sub-culture on liver agar.

For further comparative purposes a group of guinea-pigs was inoculated with a culture of Strain 45 which had been sub-cultivated 550 times since September 1931. Another group of guinea-pigs was inoculated with formalinised vaccine. Particulars of the vaccination of the groups of guinea-pigs and the test inoculation of virulent culture are as follows :—

Group 1.—Vaccinated with 6,148 million live organisms, Strain 45, Sub-culture X21.

Group 2.—Vaccinated with 6,148 million live organisms, Strain 45 passaged through six guinea-pigs, Sub-culture 3. The culture from which the vaccine was prepared consisted of rather a sparse growth, the media apparently not being up to the usual standard of quality.

Group 3.—The vaccine was in all respects comparable to that used for Group 2, except that the vaccine was prepared from a culture of very good growth.

Group 4.—Vaccinated with 6,148 million live organisms of Strain 45, Sub-culture X550.

Group 5.—Vaccinated with 6,148 million organisms of Strain 45, Sub-culture X21, sterilised by the action of 0·25 per cent. formalin acting at 37° C. for 48 hours.

Group 6.—Controls.

A test inoculation of 300 organisms was given 35 days after the inoculation of the single dose of vaccine. The strain was the same as that used in the previous experiment but the numbers of organisms used was 100 times fewer. The reduction was made because of the possibility that the test inoculation in the preceding experiment had been too severe.

The guinea-pigs were examined in equal numbers from each group at from 38 to 47 days after receiving the test inoculation. The results of the experiment are summarised in Table VII.

Accepting the absence of infection from all the organs examined as the most reliable criterion for assessing the resistance of the groups, it is evident that both Group 2 and 3, which had been vaccinated with the animal-passaged strain, were the most resistant, a comparison of these two groups with the control group showing a difference of 80 per cent. in favour of the former. The two groups 1 and 4 inoculated with vaccine prepared from Sub-culture X21 and Sub-culture X550 respectively showed a 50 per cent. greater resistance to infection than the controls, which probably reflects a definite degree of immunity produced by the vaccination. Group 5 inoculated with formalinised vaccine showed a superiority of only 20 per cent. over the controls.

Infection of the liver and kidney not being detected regularly among the controls, furnish records of limited value, as do the spleen weights. But upon whichever of these data the groups be compared, Nos. 2 and 3 are found to be the most resistant. An intermediate position is occupied by Groups 4 and 5, except that

TABLE VII.

Group.	*No. of G. Pigs Examined.*	*No. found Infected.*	*No Heavily Infected in Spleen.*	*No. Infected in Liver.*	*No. Infected in Kidney.*	*Average Weight of Spleens.*	*Remarks.*
1. Vaccinated. Str. 45, Sub. X21	10	4	1	2	1	1·32 grammes.	Two showed macroscopic lesions of the spleen.
2. Vaccinated. Passaged Str. 45	10	1	1	0	0	1·13 grammes.	None showed lesions.
3. Vaccinated. Passaged Str. 45	10	1	0	0	0	1·00 gramme.	None showed lesions.
4. Vaccinated. Str. 45, Sub. X350	10	4	1	0	1	1·43 grammes.	Two showed macroscopic lesions of the spleen.
5. Vaccinated. Str. 45, Sub. X21 Formalinised	10	7	3	3	0	2·39 grammes.	Four showed macroscopic lesions of the spleen.
6. Controls	10	9	4	1	2	1·95 grammes.	Seven showed macroscopic lesions of the spleen and three showed macroscopic lesions of one or both epididymi.

Even after animal passage the " ærobic " vaccine strain was not recovered from the guinea-pigs in Groups 2 and 3.

in the case of Group 5 the spleen weights being heavier than those shown by the controls suggests that in this particular respect Group 5 were no more resistant than the control animals and this therefore relegates Group 5 to a position subordinate to both Groups 1 and 4; hence the relative position of the three groups is again found to be the same as when resistance was assessed on the numbers of animals in each group found to be free from infection. The superior resistance of animals in Groups 2 and 3 suggests that animal passage had improved Strain 45 as a vaccine, without resuscitating virulence for these animals.

The test inoculation in this experiment was sufficiently severe to cause infection in nine out of ten control animals, but not too drastic to break through the resistance of all members in the vaccinated groups.

SUMMARY.

The immunisation of guinea-pigs inoculated with living micro-organisms of a non-virulent strain of *Br. abortus* is recorded. The strain though smooth and fully agglutinable does not stimulate the production of agglutinins in high titre even when inoculated in very large amounts. This is attributed to the lack of invasiveness and the inability of the organisms to persist in the body of the inoculated animal and not to defective or deficient antigenic structure.

Female guinea-pigs when pregnant were found highly susceptible to a uterine infection with *Br. abortus*, death of the fœtus or fœtuses and abortion frequently occurring. After abortion, abscessation of the uterus was sometimes encountered and it is regarded as specific evidence of a uterine infection with *Br. abortus*. Female guinea-pigs vaccinated with the non-virulent strain before pregnancy and infected whilst pregnant or when running with male animals, though infected in the spleen, were shown to possess a high degree of resistance to a uterine infection which was not enjoyed by control animals. Male guinea-pigs inoculated with either single or multiple large doses of the non-virulent living organisms showed in some experiments a markedly increased resistance to infection, enabling the results of the experiments to be judged by a comparison of the number of infected vaccinated animals with the number of infected control animals, to the decided advantage of the former. Evidence was obtained suggesting that vaccinated animals, though not in all cases completely resistant to an infection, showed very considerable resistance to a second or superimposed infection. This does not, however, indicate that immunity necessarily depends upon the persistence of infection. Guinea-pigs inoculated with the non-virulent strain were found to be resistant after a period of 19 weeks, the longest period tested. As there is evidence to show that the non-virulent strain rarely persists in the body of an inoculated

guinea-pig for more than three weeks, an immunity lasting for 19 weeks may be regarded as one which is independent of the persistence of infection in the body.

Comparative tests of vaccine prepared from different sub-cultures of the non-pathogenic strain, which were separated by over 200 sub-cultivations in one instance and by more than 500 in the other, do not indicate any definite antigenic deterioration caused by repeated sub-cultivation alone.

The non-virulent strain after " intermittent " passage through a series of six guinea-pigs was still non-pathogenic, but there was some evidence that as a vaccine it was superior to the non-passaged strain. The evidence is too limited to warrant any conclusion, but it indicates one direction in which further experiments should be directed.

Although in the majority of experiments clear-cut results were obtained this was not always the case. Why in a few instances vaccinated groups of guinea-pigs showed little or no more resistance than the control animals is still obscure.

The killed vaccines tested were unsatisfactory.

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Attenuation and Vaccine Potential of *aroQ* Mutants of *Corynebacterium pseudotuberculosis*

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Corynebacterium pseudotuberculosis*, a gram-positive intracellular bacterial pathogen, is the etiological agent of the disease caseous lymphadenitis (CLA) in both sheep and goats. Attenuated mutants of *C. pseudotuberculosis* have the potential to act as novel live veterinary vaccine vectors. We have cloned and sequenced the *aroB* and *aroQ* genes from *C. pseudotuberculosis* C231. By allelic exchange, *aroQ* mutants of both C231, designated CS100, and a *pld* mutant strain TB521, designated CS200, were constructed. Infection of BALB/c mice indicated that introduction of the *aroQ* mutation into C231 and TB521 attenuated both strains. In sublethally infected BALB/c mice, both CS100 and CS200 were cleared from spleens and livers by day 8 postinfection. The in vivo persistence of these strains was increased when the intact *aroQ* gene was supplied on a plasmid in *trans*. Mice infected with TB521 harbored bacteria in organs at least till day 8 postinfection without ill effect. When used as a vaccine, only the maximum tolerated dose of CS100 had the capacity to protect mice from homologous challenge. Vaccination with TB521 also elicited protective immunity, and this was associated with gamma interferon (IFN-γ) production from splenocytes stimulated 7 days postvaccination. The role of IFN-γ in controlling primary infections with *C. pseudotuberculosis* was examined in mice deficient for the IFN-γ receptor (IFN-γR$^{-/-}$ mice). IFN-γR$^{-/-}$ mice cleared an infection with CS100 but were significantly more susceptible than control littermates to infection with C231 or TB521. These studies support an important role for IFN-γ in control of primary *C. pseudotuberculosis* infections and indicate that *aroQ* mutants remain attenuated even in immunocompromised animals. This is the first report of an *aroQ* mutant of a bacterial pathogen, and the results may have implications for the construction of aromatic mutants of *Mycobacterium tuberculosis* for use as vaccines.

Corynebacterium pseudotuberculosis, a gram-positive facultative intracellular pathogen, is the etiological agent of caseous lymphadenitis (CLA) in sheep and goats. CLA is a chronic disease typically characterized by necrotizing inflammation of one or more superficial lymph nodes. In sheep, CLA results in reduced wool production and meat losses due to carcass condemnation (31). In Australia, it is one of the most prevalent diseases of sheep, with economic losses in the order of $20 million per year (30). While the pathogenic process employed by *C. pseudotuberculosis* in causing CLA in sheep and goats is not well defined, at least two major virulence determinants have been identified. One of these is the toxic lipid cell wall, which may mediate the bacterium's resistance to killing by phagocytic cells (10). The other identified virulence determinant is a sphingomyelin-degrading phospholipase D (Pld) exotoxin (22). Pld is thought to mediate dissemination of the pathogen within the host by increasing local vascular permeability (3). A role for Pld in the virulence of *C. pseudotuberculosis* was confirmed when an isogenic *pld* mutant was constructed and shown to be unable to cause CLA. Importantly, sheep immunized with a *pld* mutant were protected from subsequent challenge with the wild-type parental strain (13). This Δ*pld* mutant holds promise as a veterinary vaccine vector, since it is capable of eliciting immune responses to coexpressed antigens in vaccinated sheep (14). There is, however, accumulating evidence to suggest that the type of mutation used to attenuate a vaccine vector can have a critical influence on the vector's ability to elicit an immune response to a carried foreign antigen (23). This most probably reflects the altered in vivo growth rate or persistence of the pathogen and coincident altered interaction with the immune system.

Several different bacterial pathogens have been attenuated by stable introduction of mutations in the aromatic amino acid biosynthetic pathway. Aromatic-dependent mutants of the following pathogens have been shown to be attenuated and capable of stimulating protective immunity in different animal models: *Salmonella typhimurium* (17), *Salmonella typhi* (26), *Salmonella cholerasuis* (27) *Shigella flexneri* (43), *Bordetella pertussis* (36), *Pasteurella multocida* (18), *Bacillus anthracis* (20), *Aeromonas salmonicida* (42), *Yersinia enterocolitica* (4), and *Yersinia pestis* (29). The reduced virulence of these bacterial strains is likely to be due to their requirement for *p*-aminobenzoic acid, a precursor of folic acid and a compound which is not synthesized by chordates. Since bacteria are unable to take up exogenous folate and the availability of *p*-aminobenzoic acid is limited in vertebrate tissues, the growth of *aro* mutants in vivo is severely restricted.

Here we report the cloning of the *aroB* and *aroQ* genes from *C. pseudotuberculosis* and their similarity with the corresponding genes from *Mycobacterium tuberculosis*. An *aroQ* mutant of *C. pseudotuberculosis* was constructed by allelic exchange, and experiments were conducted to test its efficacy as a vaccine in a murine model of infection. We believe this to be the first rationally attenuated prechorismate *aro* mutant of a gram-positive bacterium. The construction of an aromatic mutant of

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TABLE 1. Bacterial strains and plasmids used in this present study

Strain or plasmid	Relevant characteristics	Reference or source
C. pseudotuberculosis		
C231	Wild type	13
TB521	*pld* mutant of C231; generated by in vitro site-directed mutagenesis of *pld* sequence encoding Pld active site (His20→Ser20), then allelic exchange with wild-type gene in C231	41 and unpublished data
CS100	C231 *aroQ*::*erm*	This study
CS200	TB521 *aroQ*::*erm*	This study
E. coli		
AB2829	K-12 *aroA* mutant	A. J. Pittard (32)
AB2826	K-12 *aroB* mutant	A. J. Pittard (32)
AB2830	K-12 *aroC* mutant	A. J. Pittard (32)
AB1360	K-12 *aroD* mutant	A. J. Pittard (32)
BRD728	Lambda lysogen	Gift from G. Dougan
JM101	Cloning host	
DH5α	Cloning host	
Plasmids		
pHC79	Cosmid cloning vector	16
pBluescript	Cloning vector	Stratagene, La Jolla, Calif.
pEP-2	*E. coli-C. pseudotuberculosis* shuttle vector	34
pUC4K	Source of *Kan* cassette	Pharmacia, Piscataway, N.J.
pBTB24	Source of *erm* cassette	15
pCS1	*aroB*/*aroD* complementing cosmid	This study
pCS2	3-kb *Bam*HI fragment from pCS1 containing *aroB* and *aroQ* genes	This study
pCS3	3-kb *Bam*HI fragment from pCS2 blunt-end ligated to *Pvu*II-digested pBluescript	This study
pCS4	*kan* gene blunt-end ligated to *Sca*I-digested pCS3	This study
pCS5	*kan* gene blunt-end ligated to *Sca*I-digested pBluescript	This study
pCS6	*erm* gene blunt-end ligated to *Eco*RI-digested pCS4	This study
pCS7	*erm* gene blunt-end ligated to *Pvu*II-digested pCS5	This study
pCS8	3-kb *Bam*HI fragment from pCS2 in pEP-2	This study

C. pseudotuberculosis will facilitate an immunobiological comparison with the previously constructed Δ*pld* mutant with respect to efficacy as a CLA vaccine and also as a vaccine vector.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The properties of the bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were routinely grown in Luria-Bertani (LB) broth or agar supplemented with ampicillin (50 μg/ml), kanamycin (50 μg/ml), or erythromycin (200 μg/ml) when appropriate. M9 minimal medium (37) containing ampicillin and essential nonaromatic amino acids was used to select for cosmid clones which complemented the *E. coli* aromatic mutants. *C. pseudotuberculosis* strains were cultured in brain heart infusion (BHI); (Oxoid, Basingstoke, Hampshire, England) broth or agar for 2 days at 37°C. When appropriate, the medium was supplemented with erythromycin (150 ng/ml) or kanamycin (50 μg/ml).

DNA manipulation and analyses. Plasmid DNA preparation, restriction enzyme digests, DNA hybridization, ligations, and transformations were performed by using standard techniques (37). DNA fragments were purified from agarose gels by using Geneclean (Bio 101 Inc., Vista, Calif.). DNA probes were prepared by nick translation using [α-^{32}P]dATP (Amersham International, Buckinghamshire, England) according to the manufacturer's instructions. Similarly, DNA probes were stripped from Hybond-N (Amersham) membranes according to the manufacturer's instructions. DNA sequencing was performed by the dideoxy-chain termination method, using fluorescein-labeled dideoxynucleotides. The DNA sequence was analyzed with an Applied Biosystems 373A DNA sequencer (Perkin-Elmer, Melbourne, Victoria, Australia). Specific synthetic oligonucleotides and commercial (Promega Corp., Madison, Wis.) universal and reverse oligonucleotides were used in sequencing. C231 genomic DNA for construction of the cosmid library was a kind gift from Catherine Pogson (CSIRO Division of Animal Health). Genomic DNA from *C. pseudotuberculosis* was isolated by the whole-cell lysate technique (44).

Construction of a cosmid genomic library. C231 genomic DNA was partially digested with *Sau*3A and fractionated on a 10 to 40% sucrose gradient. DNA fragments in the 35- to 50-kb range were ligated to the cosmid vector pHC79, digested with *Bam*HI, and dephosphorylated with calf intestinal alkaline phosphatase. Aliquots of the ligation were packaged in vitro into bacteriophage lambda, using a commercial packaging kit (Boehringer GmbH, Mannheim, Germany).

Packaged cosmid clones were transduced into *E. coli* BRD728, which harbors a defective bacteriophage lambda lysogen stably maintained at 30°C. Cells were prepared for transduction according to the method supplied with the packaging kit. The library was amplified essentially as described by Jacobs et al. (21) and stored under chloroform at 4°C.

Complementation analysis. Repackaged recombinant cosmid molecules were transduced into cells of the particular *E. coli* aromatic auxotroph, prepared as described above for bacteriophage lambda infection, at a multiplicity of infection of 0.1. After a 30-min absorption period, 1 ml of LB broth was added and the cells were incubated at 37°C for 45 min to allow expression of antibiotic resistance genes. Cells were then washed twice with saline before being plated onto M9 minimal medium containing ampicillin.

Construction of aromatic mutants by allelic exchange. A suicide plasmid construct which could mediate allelic exchange and the generation of an *aroQ* mutant of *C. pseudotuberculosis* was constructed. The 3-kb *Bam*HI fragment in pCS2 was excised with *Bam*HI, made blunt-ended by using the Klenow fragment of DNA polymerase, and ligated to *Pvu*II-digested pBluescript KS$^+$ to produce pCS3. The *Eco*RI site 324 bp downstream from the putative ATG of the *aroQ* open reading frame was now unique in pCS3. The unique *Sca*I site located in the ampicillin resistance gene in pBluescript was then used. A *Hin*cII fragment containing the kanamycin resistance cassette from pUC4K was blunt-end ligated to *Sca*I-digested pCS3 and also to *Sca*I-digested pBluescript, generating pCS4 and pCS5, respectively. Finally, the *aroQ* gene was insertionally inactivated by blunt-end ligation of an erythromycin resistance cassette from pBTB24 to the blunt-ended (Klenow fragment) unique *Eco*RI site within the *aroQ* open reading frame in pCS4 to generate pCS6. The same erythromycin resistance cassette was also ligated to *Pvu*II-digested pCS5 to generate pCS7. Thus, pCS7 is identical to pCS6 but lacks the 3-kb *Bam*HI fragment harboring the *aroB* and -*Q* genes. pCS7 was used as a control to screen for the frequency of illegitimate recombination of plasmid sequences with the *C. pseudotuberculosis* chromosome. *C. pseudotuberculosis* C231 and pTB521 were electroporated with 5 μg of the appropriate plasmid construct (pCS6 or pCS7). Following electroporation, putative *aroQ* mutants were selected on BHI plates containing 150 ng of erythromycin per ml for 4 days at 37°C. Erythromycin-resistant colonies were presumed to result from a recombination event. Bacteria which had undergone an allelic exchange event whereby the entire plasmid had been integrated (merodiploid) would also be kanamycin resistant. Erythromycin-resistant colonies were patched onto BHI agar containing kanamycin. Erythromycin-resistant, kanamycin-sensitive colonies were subsequently analyzed by Southern hybridization for an allelic replacement event at the *aroQ* locus.

LD_{50} experiments and in vivo growth. BALB/c mice were pedigree bred and maintained in the Department of Microbiology, University of Melbourne, Melbourne, Parkville, Australia. For 50% lethal dose (LD_{50}) experiments, groups of five sex- and age-matched mice were infected intraperitoneally with serial 10-fold dilutions of *C. pseudotuberculosis* in saline. The infecting dose was calculated retrospectively by viable count on BHI agar. Mice were killed by cervical dislocation when moribund. The LD_{50} value was calculated by the method of Reed and Muench (35) at the end of 8 weeks. The kinetics of bacterial growth in vivo was evaluated by sacrificing groups of four mice at regular time intervals postinfection. Spleens and livers were homogenized in a blender (Stomacher 80) Seward Medical, London, England), and the bacterial load was enumerated by viable count on BHI agar.

129/Sv/Ev mice of either sex homozygous for a disrupted gamma interferon receptor (IFN-γR) gene (IFN-$\gamma R^{-/-}$) and for null mutation (IFN-$\gamma R^{+/+}$) were produced as described previously (19) and bred at the John Curtin School of Medical Research, Australian National University, Canberra, Australia.

Immunization and wild-type challenge. Groups of 12 BALB/c mice were immunized intraperitoneally with 10-fold serial doses of CS100, TB521, or heat-killed bacteria. The viable count of the immunizing inoculum was determined retrospectively. Bacteria were heat killed by incubation at 60°C for 30 min. Verification of killing was determined by viable count. All mice, including naive controls, were challenged intraperitoneally 21 days postvaccination with an infectious dose of the wild-type strain. Groups of challenged mice were sacrificed on days 7 and 14 postchallenge, and organs were removed for bacterial culture.

Cytokine induction and IFN-γ ELISA. Spleens from immunized mice were removed aseptically, and single-cell suspensions were prepared by passage through wire sieves. Erythrocytes were removed by treatment with 0.017 M Tris-ammonium chloride, washed twice, and suspended in RPMI (CSL Ltd., Melbourne, Victoria, Australia) containing 10% fetal bovine serum, 2 mM L-glutamine, 1 mM pyruvate, 5×10^{-5} M β-mercaptoethanol, penicillin (100 U/ml), and streptomycin (50 μg/ml). Bulk splenocytes were seeded at 5×10^6/ml in 0.5 ml in 48-well tissue culture plates (Costar, Cambridge, Mass.). Cells were stimulated with 5 μg of soluble *C. pseudotuberculosis* cell lysate and incubated for 48 h before supernatant fluids were harvested. Control wells were stimulated with concanavalin A (5 μg/ml; Sigma, St. Louis, Mo.) or medium alone. Supernatant fluids were used in a IFN-γ cytokine enzyme-linked immunosorbent assay (ELISA) (CSL Ltd.) which was performed according to the manufacturer's instructions. The limit of detection in the assay was 2 IU/ml.

Statistics. The mean number of challenge bacteria recovered from immunized mice was compared to the number recovered from unvaccinated mice by using ordinary one-way analysis of variance with Dunett's analysis. The unpaired Student *t* test was used for comparison of bacterial counts from IFN-$\gamma R^{-/-}$ mice with control mice.

Nucleotide sequence accession number. The complete nucleotide sequence of the *C. pseudotuberculosis* C231 *aroB* and *aroQ* genes has been lodged in GenBank under accession no. U88628.

RESULTS

Cloning of genes from the prechorismate aromatic amino acid biosynthetic pathway. Initial attempts to clone the *C. pseudotuberculosis aroA* gene by shotgun cloning 2- to 4-kb *Sau*3A-digested genomic DNA fragments and identification of recombinants which could complement the *aroA E. coli* mutant AB2829 proved unsuccessful. To increase the likelihood of obtaining a representative genomic library, a cosmid library was constructed in the vector pHC79. The library was amplified in the lambda lysogen BRD728 by incubation at 37°C, and the lysate containing a cosmid bearing defective phage particles was used to independently transduce each of the prechorismate *E. coli aro* mutants AB2829, AB2826, AB2830, and AB1360. Complementation of growth on minimal medium was achieved only for the *aroB* and *aroD E. coli* mutants, AB2826 and AB1360, respectively. Restriction enzyme digests of complementing cosmids isolated from both mutants suggested the presence of many shared restriction fragments. Indeed, all analyzed cosmids that complemented AB2826 could also complement AB1360.

One such cosmid (pCS1) was subcloned to a 3-kb *Bam*HI fragment in pBluescript KS^+. The resultant construct, pCS2, retained the ability to complement growth of both AB2826 and AB1360. Further subcloning and sequencing of pCS2 identified two open reading frames. BLAST analysis of the DNA and predicted amino acid sequences of the two open reading frames indicated most significant amino acid identity with the *aroB* and *aroQ* gene products from *M. tuberculosis*. These shikimate pathway genes encode the enzymes dehydroquinate synthase and a type II 3-dehydroquinase, respectively. On the basis of sequence identities, these genes from *C. pseudotuberculosis* were designated *aroB* and *aroQ*. The deduced amino acid sequences of the *aroB* and *aroQ* genes from *C. pseudotuberculosis* were aligned with the amino acid sequences of other *aroB* and *aroQ* genes by using the CLUSTAL multiple alignment program (Australian Genomic Information Service). The deduced amino acid sequence of the *aroB* gene displayed significant identity with *aroB*-encoded enzymes from other bacterial species (Fig. 1A). The deduced amino acid sequence of the *aroQ* gene, while displaying significant identity with the *M. tuberculosis aroQ* gene product, also displayed identity with catabolic enzymes involved in quinic acid catabolism in fungi (Fig. 1B).

Construction of *C. pseudotuberculosis aroQ::erm*. The observation that ColE1-based plasmids such as pBluescript KS^+ cannot replicate in *C. pseudotuberculosis* facilitated the construction of a suicide targeting vector designed to mutate the *aroQ* gene. The construction of pCS6 (targeting vector) and pCS7 (control plasmid) is outlined in the Materials and Methods. Importantly, pCS6 could not complement the growth of *aroD E. coli* mutant AB1360 on minimal media. This result suggested that the product of the cloned *aroQ* gene was now no longer functional. Electroporation of pCS6, but not the control plasmid pCS7, into *C. pseudotuberculosis* C231 and TB521 yielded erythromycin-resistant colonies of various sizes after 4 days of incubation at 37°C. These colonies were presumed to have resulted from a recombination event. Erythromycin-resistant colonies were then patched onto BHI agar containing kanamycin. Of the erythromycin-resistant colonies generated, approximately 5% were kanamycin sensitive.

Unlike the situation with many gram-negative pathogens, the lack of a defined minimal medium for culture of *C. pseudotuberculosis* prevented screening putative *aroQ* mutants for aromatic amino acid auxotrophy. As a means of verifying allelic exchange, chromosomal DNAs from putative mutants and the wild-type strain were analyzed by Southern hybridization with probes specific for *aroB* and *-Q* and the erythromycin resistance gene. The strategy used to construct and analyze these mutants is represented schematically in Fig. 2A.

As predicted, a 3.8-kb *Bgl*II fragment in representative *aroQ* mutants of C231 and TB521 hybridized to the 2-kb *Bgl*II *aroB,Q*-specific probe (Fig. 2B). The gel shift of 1.8 kb in the mutants relative to the band in the wild-type parental strain corresponds to the size of the erythromycin resistance cassette inserted into the *aroQ* gene. After stripping of the *aroB,Q*-specific probe, an identically sized 3.8-kb *Bgl*II fragment in DNA extracted from the putative mutants hybridized to an erythromycin resistance gene-specific probe (Fig. 2C). With this probe, there was no hybridization to DNA extracted from the wild-type strain. Labeled pBluescript DNA did not hybridize to DNA from either the wild-type strain or the representative mutants (data not shown). The results indicate legitimate allelic exchange at the *aroB* and *-Q* loci, resulting in the construction of an *aroQ* mutant of C231, designated CS100, and also of TB521, designated CS200.

In vitro growth characteristics. Initial observations on the growth characteristics of CS100 and CS200 suggested that they grew more slowly in vitro than their parental counterparts. Importantly, however, the growth rate could be restored by providing the cloned *aroQ* gene on a plasmid in *trans* (Fig. 3). These results suggested that the reduced growth rate displayed by the *aroQ* mutants was not a pleiotropic effect caused by introduction of the *aroQ* mutation but rather more likely re-

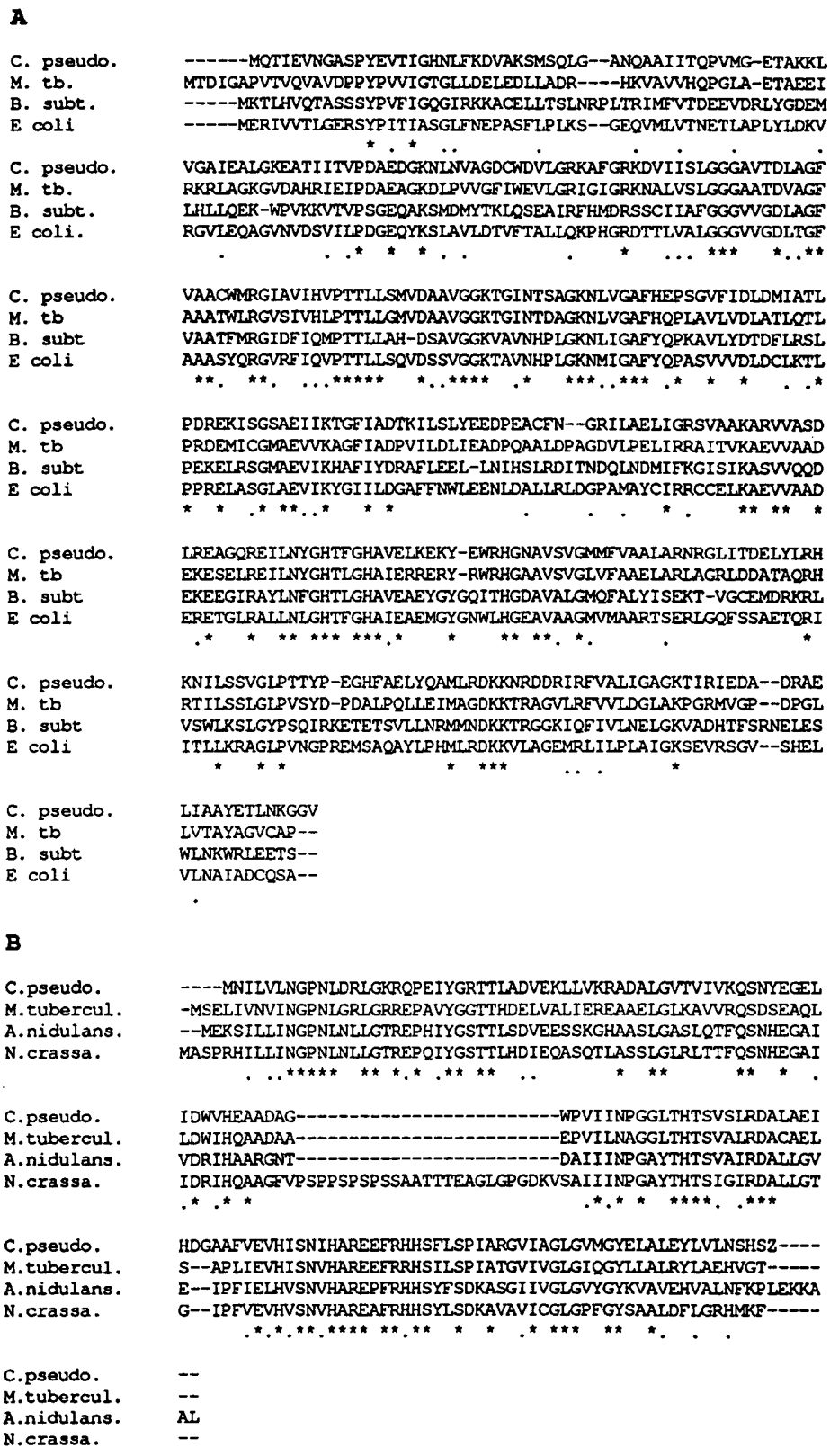

FIG. 1. Amino acid comparisons of *aroB* and *aroQ* gene products. (A) Comparison of deduced amino acid sequences of *aroB*-encoded dehydroquinate synthase enzymes from *C. pseudotuberculosis* (*C. pseudo.*), *M. tuberculosis* (*M. tb.*), *E. coli*, and *B. subtilis* (B. subt.). (B) Comparison of deduced amino acid sequences of *aroQ*-encoded 3-dehydroquinase enzymes from *C. pseudotuberculosis* and *M. tuberculosis* (M. tubercul.) in optimal alignment with the catabolic 3-dehydroquinases from *Aspergillus nidulans* and *Neurospora crassa*. The alignment was made by using the CLUSTAL DNA alignment program made available through the Australian Genomic Information Service. In each alignment, residues that are identical between species are indicated by asterisks. Conservative substitutions are indicated by dots.

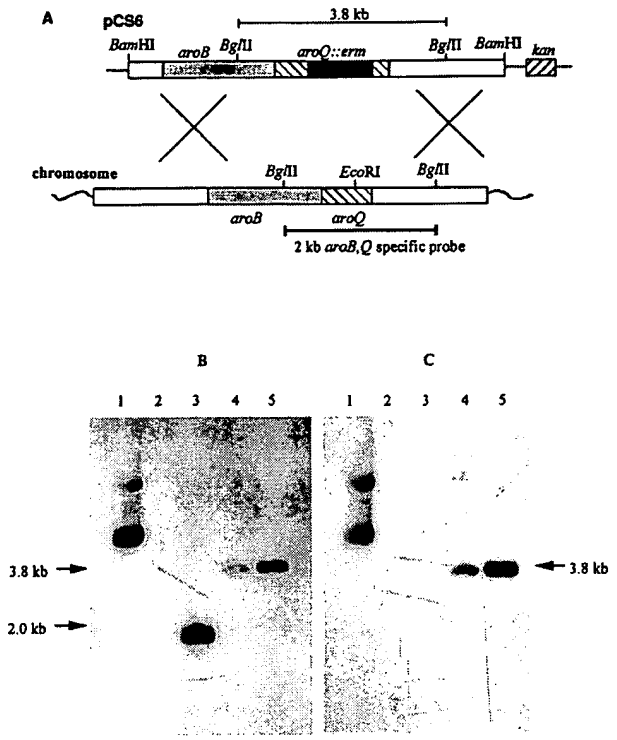

FIG. 2. The construction of *aroQ* mutants of *C. pseudotuberculosis*. (A) Schematic diagram illustrating the strategy used to generate *aroQ* mutants and derive probes for screening recombinants. (B and C) Results of Southern hybridization of chromosomal DNA extracted from C231, CS100, and CS200, digested with *Bgl*II, and probed with (B) an α-^{32}P-labeled 2-kb *Bgl*II *aroB,Q*-specific gene probe (B) and an α-^{32}P-labeled erythromycin resistance gene. Lanes: 1, uncut pCS6; 2, empty lane; 3, C231; 4, CS100; 5, CS200.

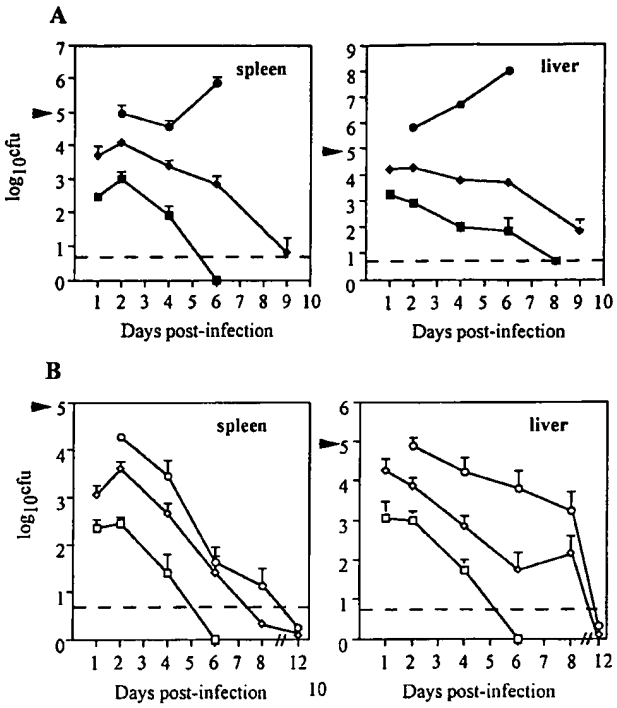

FIG. 4. *aroQ* mutants fail to persist in vivo. (A) In vivo growth of C231 (●), CS100 (■), and CS100(pCS8) (◆) in spleens and livers of BALB/c mice after intraperitoneal injection of 10^5 bacteria of each strain. (B) In vivo growth of TB521 (○), CS200 (□), and CS200(pCS8) (◇) in spleens and livers of BALB/c mice after intraperitoneal injection of 10^5 bacteria of each strain. In all cases, each point represents the mean of four mice plus the standard deviation. The detection limit (dashed line) was 5 CFU/organ.

flects the inability of *C. pseudotuberculosis* to scavenge one or more key aromatic metabolites from the growth medium.

***aroQ* mutants of *C. pseudotuberculosis* are attenuated in a mouse model of infection.** Introduction of the *aroQ* mutation into *C. pseudotuberculosis* C231 increased the LD_{50} for BALB/c mice by ~3 logs (increase in the $\log_{10}$ LD_{50} value from 2.5 to 5.3). In contrast, introduction of the *aroQ* mutation into TB521 attenuated this strain by only a further log relative to the attenuation already caused by mutation of *pld* (increase in the $\log_{10}$ LD_{50} value from 5.3 to 6.3). Mice which succumbed to infection with high doses of either *aroQ* mutant died within 24 to 48 h. However, death appeared to be associated with direct toxicity and not bacteremia, since, as determined by bacterial culture of organs, there was no significant expansion of bacterial numbers in vivo. These experiments identified the maximum tolerated dose for BALB/c mice as ~10^6 CFU.

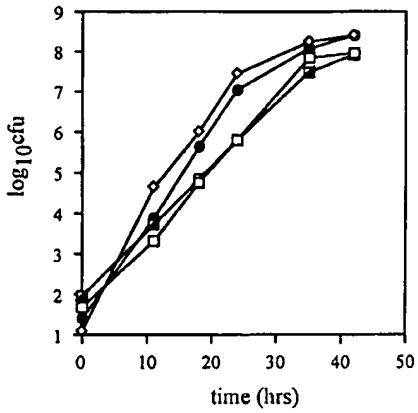

FIG. 3. In vitro growth rates of C231 (●), CS100 (□), CS100(pEP-2) (), and CS100(pCS8) (◇) in BHI broth. The results are representative of two separate experiments.

Persistence of *aro* mutants and parental counterparts in vivo. To better understand the basis for the observed attenuation, we examined the degree of in vivo persistence of CS100, CS200, and their parental strains in sublethally infected mice (Fig. 4A and B). The results demonstrated that introduction of an *aroQ* mutation severely restricted the in vivo growth of the mutants compared with their parental counterparts. Bacteria harboring an *aroQ* mutation, regardless of parental background, could not be cultured from spleens and livers of infected mice beyond 8 days postinfection. In contrast, mice infected with wild-type strain C231 harbored a significant bacterial burden in spleens and livers and were moribund by day 6 postinfection (Fig. 4A). Mice infected with TB521 harbored bacteria in spleens and livers, albeit in reduced numbers, until at least day 8 postinfection (Fig. 4B). These mice did not display any clinical symptoms. When the *aroQ* mutants were complemented in *trans* with an intact *aroQ* gene, the in vivo persistence of both CS100 and CS200 was increased but remained less than that of either parental strain.

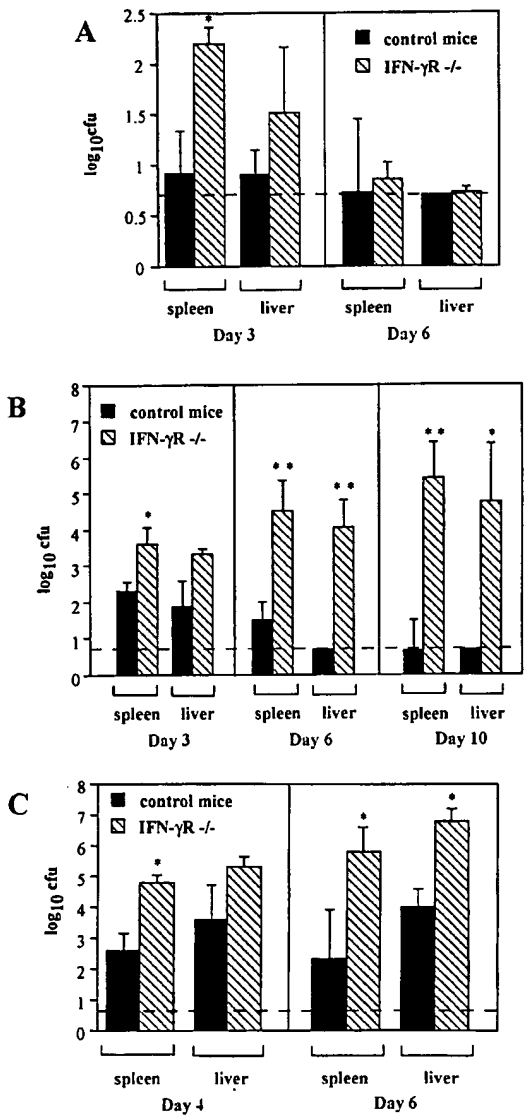

FIG. 5. In vivo growth of CS100 (A), TB521 (B), and C231 (C) in IFN-γR$^{-/-}$ and homozygous control mice. Mice were intraperitoneally infected with 5×10^4 CFU of CS100, 10^4 CFU of TB521, and 10^4 CFU of C231. Each bar represents the mean organ count from four mice plus the standard deviation. The detection limit (dashed line) was 5 CFU/organ. * denotes $P < 0.01$, and ** denotes $P < 0.001$, (Student's t test) compared to homozygous control mice.

***aroQ* mutants are attenuated in IFN-γR$^{-/-}$ mice.** A requirement of live attenuated vaccines is that they should be safe even when used in immunocompromised hosts. In relation to this, the importance of the cytokine IFN-γ in controlling growth of both intracellular bacteria and viruses is well documented. We have used gene knockout mice which lack the receptor for IFN-γ to determine whether this cytokine has a role in mediating clearance of *C. pseudotuberculosis* in vivo. At the infectious dose given, the overall kinetics of infection with CS100 in IFN-γR$^{-/-}$ mice was not greatly different from that in homozygous control mice (Fig. 5A). This result suggests that IFN-γ plays only a minor role in mediating clearance of an aromatic mutant of *C. pseudotuberculosis.*

In direct contrast, IFN-γR$^{-/-}$ mice infected with either the *pld* mutant TB521 (Fig. 5B) or the wild-type strain, C231 (Fig. 5C) were highly susceptible to infection compared to homozygous control mice. As a control for the phenotype of these mice, animals were also infected with an *S. typhimurium* Δ*aroA* Δ*aroD* mutant (BRD509); in accordance with published results (12), IFN-γR$^{-/-}$ mice were more susceptible to infection than control mice (data not shown).

Protection against *C. pseudotuberculosis* wild-type challenge. Groups of six BALB/c mice were immunized intraperitoneally with graded sublethal doses of either CS100, TB521, or heat-killed CS100. All mice, including naive controls, were subsequently challenged 21 days postvaccination with an infectious dose (5×10^3 CFU) of the wild-type strain. Groups of challenged mice were sacrificed on days 7 and 14 postchallenge, and organs were removed for bacterial culture of the wild-type strain (Fig. 6). Protection was assessed by comparison of the bacterial load in naive mice to that in immunized mice. At 14 days postchallenge, the absence of challenge bacteria in the organs of mice immunized with 5×10^5 or 5×10^4 CFU of TB521 indicated the development of protective immunity in all of these animals. Similarly, mice immunized with the maximum tolerated dose of CS100 (8×10^5 CFU) harbored significantly fewer challenge bacteria at both days 7 and 14 postchallenge compared to naive animals. In contrast, mice which received 8×10^4 CFU of CS100, 1×10^6 CFU of heat-killed bacteria, or no vaccine were not protected from challenge and harbored significant bacterial burdens in both the spleen and liver at days 7 and 14 postinfection.

Induction of IFN-γ by vaccine strains. At day 7 postvaccination, IFN-γ could be detected in supernatant fluids of stimulated splenocytes derived from mice vaccinated with 8×10^5 CFU of CS100 or either dose of TB521. Thus, there was a qualitative correlation between detectable IFN-γ production

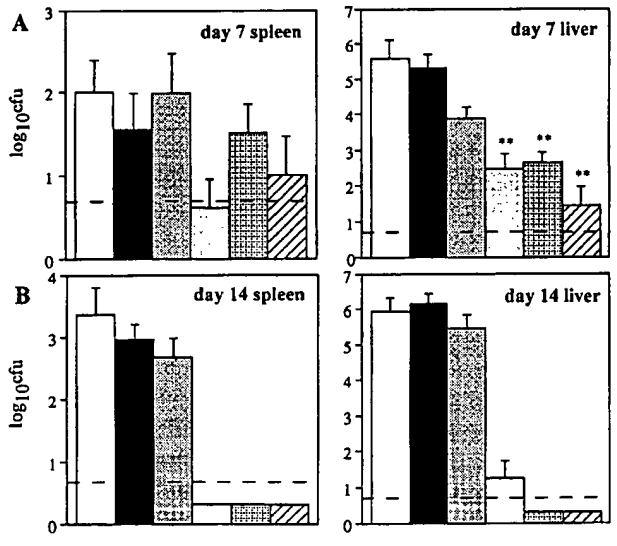

FIG. 6. Capacity of attenuated mutants of *C. pseudotuberculosis* to protect mice from a lethal homologous challenge. Groups of six mice were not vaccinated (□), or vaccinated intraperitoneally at day 0 with 10^6 heat-killed CS100 (■), 8×10^4 CFU of CS100 (▩), 8×10^5 CFU of CS100 (▨), 5×10^4 CFU of TB521 (▦), or 5×10^5 CFU of TB521 (▨). by the same route at day 21 with 5×10^3 CFU of the wild-type strain. Groups of mice were sacrificed, and organs were removed for bacterial culture of the challenge strain on day 7 (A) and day 14 (B) postchallenge. Each bar represents the mean organ count from spleens and livers of six mice plus the standard deviation. The detection limit (dashed line) was 5 CFU/organ. ** denotes $P < 0.01$ (analysis of variance) compared to unvaccinated mice.

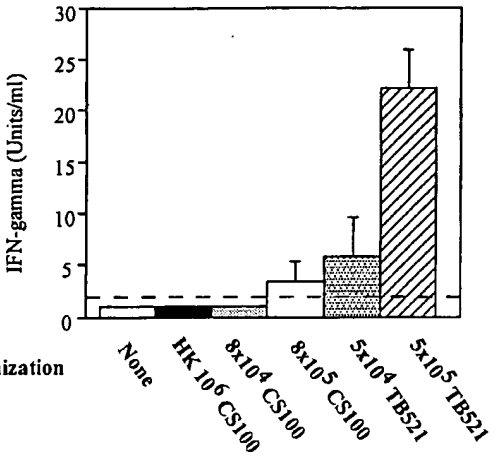

FIG. 7. IFN-γ detection in supernatant fluids of cultured mouse splenocytes isolated and pooled from vaccinated mice. Splenocytes were prepared 7 days postvaccination and stimulated with 5 μg of *C. pseudotuberculosis* soluble antigen. The detection limit of the cytokine ELISA was 2 IU/ml (dashed line).

by antigen-stimulated splenocytes derived from vaccinated mice and subsequent protection from challenge (Fig. 7). IFN-γ was not detected in supernatant fluids from stimulated splenocytes at day 14 postvaccination.

DISCUSSION

In this report, we describe the construction of *C. pseudotuberculosis* strains having insertion mutations in the *aroQ* gene and subsequent preliminary in vivo characterization. While *aro* mutants of other gram-positive bacteria have been constructed by transposon mutagenesis (1, 20), this is, to our knowledge, the first report of a rationally attenuated *aro* mutant of a gram-positive bacterial pathogen generated through allelic exchange.

Historically, *aro* genes from pathogenic bacteria have been cloned by complementation of growth of defined *E. coli aro* mutants on minimal media. This complementation approach has been highly successful in obtaining genes encoding aromatic biosynthetic enzymes from gram-negative bacterial pathogens. Using this same approach, we were able to identify cosmids of *C. pseudotuberculosis* DNA which complemented *aroB* and *aroD E. coli* mutants but not cosmids which complemented *aroA* or *aroC* mutants. This may reflect the potentially nonrepresentative nature of the cosmid library. Alternatively, it may reflect a significant degree of divergence between the *E. coli aroA*- and *aroC*-encoded enzymes and the aromatic pathway enzymes from *C. pseudotuberculosis*.

The sequential genetic arrangement of *aroB* and *aroQ* in *C. pseudotuberculosis*, as established in this study, corresponds to the order in which homologous genes are found in the taxonomically related *M. tuberculosis* (7). Moreover, the significant identity at the deduced amino acid level between the products of the *C. pseudotuberculosis* housekeeping genes *aroB*, *aroQ*, and *recA* (33), the *groEL* and *dnaK* gene products (unpublished data), and the corresponding gene products from *M. tuberculosis* confirms their relatedness at the molecular level.

Despite the amino acid sequence relatedness of the *C. pseudotuberculosis aroQ*-encoded 3-dehydroquinase enzyme to fungal catabolic enzymes from *Neurospora crassa* (8) and *Aspergillus nidulans* (11), there are several lines of reasoning in support of its involvement in the *C. pseudotuberculosis* aromatic amino acid biosynthetic pathway. First, of the analyzed recombinant cosmids and subclones capable of complementing growth of the *aroD E. coli* mutant on minimal media, virtually all displayed identical restriction enzyme digest patterns, suggesting that there is only one gene encoding a 3-dehydroquinase enzyme in *C. pseudotuberculosis*. Second, the close genetic linkage of the *aroQ* gene to the *aroB* gene, which, in *E. coli*, encodes an enzyme catalyzing the preceding step of the aromatic biosynthetic pathway, similarly suggests a biosynthetic function for the *aroQ* gene product. Third, there is evidence that *aroQ* homologs from other procaryotes, including the closely related *M. tuberculosis*, do not encode enzymes with catabolic activity (7, 25).

Random recombination of transformed DNA, as occurs in slow-growing mycobacteria, does not appear to be an impediment to the construction of mutants of *C. pseudotuberculosis* by allelic exchange. The construction of *aroQ* mutants of *C. pseudotuberculosis* was, however, problematic, since such mutants unexpectedly displayed a reduced growth rate in vitro on complex media. Importantly, however, the in vitro growth rate could be restored to wild-type levels by introduction of the intact *aroQ* gene in trans. The *aroQ* mutants generated in this study were significantly attenuated in a murine model of infection. *C. pseudotuberculosis aroQ* mutants CS100 and CS200 were cleared from livers and spleens of intraperitoneally infected mice by day 8 postinfection. While complementation in *trans* with an intact *aroQ* gene increased their in vivo persistence, it did not fully restore virulence to wild-type levels. This may, in part, have been due to plasmid segregation in vivo. Mice infected with a lethal dose of either *aroQ* mutant died within 48 h; there was not a significant expansion of bacterial numbers in either the spleen or liver, which suggested that toxicity was the cause of death.

The in vivo growth kinetics of *C. pseudotuberculosis aroQ* mutants in BALB/C mice contrasts with the behavior of *S. typhimurium aroA* mutants, which can persist in vivo for several weeks after intravenous infection (28). However, it would appear that *S. typhimurium aroA* mutants are exceptional with respect to their in vivo persistence, since *aroA* mutants of *B. pertussis* (36), *A. salmonicida* (42), *P. multocida* (18), and *Y. enterocolitica* (4) are also rapidly cleared from major organs of experimentally infected animals. Indeed, the capacity of aromatic mutants of *S. typhimurium* to kill IFN-γR$^{-/-}$ mice suggests that aromatic metabolites are not limiting in vivo and that bacterial clearance is at least partially dependent on the host's immune response. That *aroQ* mutants of *C. pseudotuberculosis* were attenuated in IFN-γR$^{-/-}$ mice suggests, not surprisingly, fundamental differences in bacterial physiology and pathogenesis between *S. typhimurium* and *C. pseudotuberculosis*.

Given the relatedness of *C. pseudotuberculosis* to *M. tuberculosis*, it is interesting to speculate on the likely phenotype of *aro* mutants of *M. tuberculosis*. The prechorismate genes *aroA*, *aroB*, and *aroQ* have been cloned from *M. tuberculosis* and represent suitable targets for mutagenesis. In light of our findings, we predict that should rational *aro* mutants of *M. tuberculosis* be constructed, they will be highly attenuated. By using cosmid DNA to mediate site-specific allelic exchange, the construction of defined mutants of *M. tuberculosis* strains now appears possible (2).

The immune mechanisms mediating clearance of CS100 in vivo remain undefined. While IFN-γR$^{-/-}$ mice have the capacity to mount a Th1-type T-cell response, macrophage activation is largely abrogated (19, 38, 39). Since IFN-γR$^{-/-}$ mice adequately controlled infections with CS100, bacterial clearance of CS100 may not be critically dependent on IFN-γ-activated macrophages. The absence of activated macrophages

does not, however, preclude the induction of acquired immunity via the two primary sources of IFN-γ, T lymphocytes and NK cells. Importantly, from a vaccine vector point of view, the results suggest that infection with CS100 is largely self-limiting, even in immunocompromised animals. On the other hand, IFN-γR$^{-/-}$ mice were highly susceptible to primary infections with either *C. pseudotuberculosis* C231 or the *pld* mutant TB521 compared to control mice. Thus, control of these primary *C. pseudotuberculosis* infections, as with infections caused by other intracellular bacterial pathogens, is significantly dependent on the bacteriocidal capacity of activated macrophages to contain and destroy the pathogen. Concordantly, Hard (9) has shown a role for T lymphocytes and activated macrophages in suppression of *C. pseudotuberculosis* growth and lesion development in a murine model.

Despite the lower level of in vivo persistence of CS100 than of TB521, this strain retained the capacity to elicit a protective immune response in BALB/c mice. The induction of protective immunity was dose dependent, however, since mice immunized with 8×10^4 CFU of CS100 were not protected from challenge. In these experiments, the induction of protective immunity by TB521 was not dose dependent, since mice immunized with either 5×10^5 or 5×10^4 CFU of TB521 were completely protected. Thus, we hypothesize that the enhanced capacity of TB521 to elicit a protective immune response can be attributed to its greater in vivo persistence. As a consequence, this strain may have an increased capacity to stimulate IFN-γ-producing T cells and/or NK cells. Indeed, appropriate T-cell stimulation is considered an essential requirement for acquired resistance against most intracellular pathogens (24). The observation that mice immunized with 10^6 heat-killed *C. pseudotuberculosis* were not protected from challenge supports the contention that a live vaccine is better able to stimulate the appropriate protective immune response. Indeed, with one exception (40), the use of killed bacterial preparations as vaccines against intracellular bacterial pathogens has historically been relatively unsuccessful (5, 6).

The role of the humoral immune response in mediating acquired resistance to *C. pseudotuberculosis* challenge remains incompletely defined. At the time of challenge, antibodies to *C. pseudotuberculosis* whole-cell lysate could not be detected in any CS100-vaccinated mice (data not shown), despite the immune status of these mice. This finding suggests that the presence of pathogen-specific circulating antibodies is not essential for protection against *C. pseudotuberculosis* challenge in a mouse model.

The use of live, rationally attenuated bacterial pathogens as vaccines, and as vaccine vectors, represents an attractive means of relatively safe, cheap, long-lasting, and efficacious vaccination, particularly within a veterinary context. We envisage that an *aroQ* mutant of *C. pseudotuberculosis*, as described here, represents a potential live vaccine and/or vaccine vector in sheep, since significantly more bacteria (up to 10^{10}) can be administered than in mice. Studies addressing these issues are currently in progress.

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Cross protection of mice and swine inoculated with culture filtrate of attenuated *Erysipelothrix rhusiopathiae* and challenge exposed to strains of various serovars

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SUMMARY

Mice and swine inoculated subcutaneously with culture filtrate vaccine prepared from acriflavine-fast attenuated *Erysipelothrix rhusiopathiae* strain Koganei 65-0.15 (serovar 2), were challenge exposed to 20 pathogenic strains of *E rhusiopathiae* of 18 serovars and type N. Vaccinated mice survived after challenge exposure to serovars 1b, 2, 8 (strain Goda), and type N, but mortality occurred in vaccinated mice challenge exposed to other strains: 20% to 30% mortality in mice challenge exposed to serovars 1a, 11, 12, 15, 16, or 21; 40% to 50% mortality in mice challenge exposed to serovars 4, 5, 6, 7, or 8 (strain 911); and 60% to 80% mortality in mice challenge exposed to serovars 9, 10, 18, or 19. All vaccinated mice died after challenge exposure with strain 2553 (serovar 20). Nonvaccinated control mice died after challenge exposure to all strains.

Of 2 vaccinated swine challenge exposed to strain 2553, 1 developed a local urticarial lesion at the site of intradermal exposure. Vaccinated swine challenge exposed to serovars 1a, 1b, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 16, 18, 19, 21, or type N did not have clinical signs of acute erysipelas. Nonvaccinated control swine developed acute generalized erysipelas or localized urticarial lesions at the site of intradermal exposure.

Strains of *Erysipelothrix rhusiopathiae* are classified into 22 serovars and type N. These serovars are based on antigenic characteristics of soluble peptidoglycans.[1,2] Most isolants of *E rhusiopathiae* from swine with clinical erysipelas are serovars 1a, 1b, and 2.[3-8] However, some isolants of relatively rare serovars 3, 4, 5, 6, 7, 8, 10, 11, 15, and 21 and type N have been obtained from swine with septicemia, urticaria, arthritis, lymphadenitis, and endocarditis.[5-8]

Some isolants of serovars 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 15, 17, 18, 19, and 20 and type N are pathogenic for swine,[9-13] indicating they could be a cause of clinical erysipelas.

Strains of *E rhusiopathiae* serovar 9 or 10 caused acute localized or generalized urticarial lesions in swine and fatal septicemia in mice vaccinated with standard erysipelas adsorbate bacterin prepared from strains of serovar 2.[14,15] Some swine vaccinated with live *Erysipelothrix* vaccine[16,17] prepared from an attenuated strain Koganei 65-0.15[18] of serovar 2 developed localized urticarial lesions at the site of intradermal exposure with serovars 8, 9, 10, or 20. There was a similarity of protection pattern in swine vaccinated with the live-organism vaccine[16,17] to that in swine vaccinated with the adsorbate bacterin,[14,15] and a specific lack of immunity induced by the live *Erysipelothrix* vaccine to certain strains of *E rhusiopathiae* in swine and mice. Seemingly, immunity induced by live-organism vaccine may be caused by specific antigenic stimulation, rather than nonspecific cellular response.

The soluble immunizing antigen(s)[19,20] in whole culture erysipelas bacterins have been described as glycolipoprotein complexes.[21] Most of the antigen is found in the culture filtrate (CF).[22] Culture filtrate of strain Koganei 65-0.15 was effective for protection of mice and swine from challenge exposure with a virulent strain of serovar 1a.[a] A cross-protective effect of CF has been demonstrated against *E rhusiopathiae* strains of serovars A and B (presently serovars 1 and 2) in mice.[21] The purpose of the present report was to determine whether CF prepared from broth culture of an attenuated strain of *E rhusiopathiae* induced cross protection in swine and mice similar to that induced by live-organism vaccine or adsorbate bacterin.

Materials and Methods

Bacterial strains—Strain Koganei 65-0.15[18] (serovar 2) of acriflavine-fast attenuated *E rhusiopathiae* was used for the preparation of CF. Twenty strains of *E rhusiopathiae* representing serovars 1 to 21 and type N (Table 1) were used for challenge exposure of mice and swine. Most of these strains originated from swine; however, 1 strain (Goda) of serovar 8 was the only strain pathogenic for swine among our collections of strains originating from birds and fish. For the determination of LD_{50},[23] mortality of mice was recorded 14 days after subcutaneous (SC) inoculation with 0.1 ml of serial 10-fold dilutions of 24-hour beef infusion broth (BIB) culture of each strain. Two pigs were inoculated intradermally with 0.1 ml of the broth culture of each strain. Each inoculum contained about 10^6 or 10^7 viable bacteria. The strain that induced a local urticarial lesion (≥ 20 mm diameter) or clinical signs of generalized erysipelas in swine within 14 days after inoculation, was recorded as pathogenic for swine.

Preparation of CF vaccine—Frozen cultures of strain Koganei 65-0.15, which had been prepared from the lyophilized stock, were thawed and cultured in BIB (pH 7.6) at 37 C for 24 hours. Beef infusion broth (3 L) containing 0.1% Tween 80[b] was inoc-

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[b] Wako Pure Chemical Industries Ltd, Osaka, Japan.

TABLE 1—Cross-protective effect of culture filtrate in mice to *Erysipelothrix rhusiopathiae* strains of various serovars

Serovar	Strain	Origin	Challenge inoculum: LD_{50} (CFU)	Challenge inoculum: Dose ($\times 10^8$ CFU)	No. of susceptible*/10 challenge exposed: Vaccinated	No. of susceptible*/10 challenge exposed: Controls
1a	Fujisawa†	Swine (septicemia)	4.38	6.04	2	10
1b	H-12†	Swine (endocarditis)	1.35	6.80	0	10
2	Saitama-1‡	Swine (urticaria)	2.70	7.54	0	10
4	2229§	Swine (spleen)	≤ 5.00	1.73	4	10
5	Pécs 67†	Swine (tonsil)	≤ 3.79	1.21	5	10
6	S-17‡	Swine (arthritis)	1.00	4.77	5	10
7	T-334‡	Swine (tonsil)	7.93	6.60	5	10
8	Goda†	Bird	14.00	38.70	0	10
8	911‡	Swine (lymphadenitis)	1.48	5.50	5	10
9	14B§	Swine pen soil	≤ 3.47	2.01	6	10
10	2179§	Swine (spleen)	0.79	1.59	8	10
11	IV 12/8†	Swine (tonsil)	2.34	8.40	3	10
12	Pécs 9†	Swine (tonsil)	22.00	61.00	2	10
15	Pécs 3597†	Swine (tonsil)	4.60	6.70	3	10
16	T-184‡	Swine (tonsil)	0.63	2.26	2	10
18	715§	Swine (spleen)	26.20	85.00	8	10
19	2017§	Swine (spleen)	22.00	50.00	7	10
20	2553§	Swine (spleen)	3.57	6.70	10	10
21	Bâno 36‖	Sheep dip	22.00	59.00	2	10
N	S-12‡	Swine (lymphadenitis)	4.26	8.50	0	10

* Susceptible = dead within 14 days after challenge exposure. † Obtained from Dr. K. Hashimoto, National Institute of Animal Health, Japan. ‡ Obtained from National Veterinary Assay Laboratory, Japan. § Obtained from Dr. R. L. Wood, National Animal Disease Center, Ames, Iowa. ‖ Obtained from Dr. V. Nørrung, State Veterinary Serum Laboratory, Copenhagen, Denmark.
CFU = colony-forming units.

ulated with 30 ml of the subculture and was incubated at 37 C for 24 hours. The number of viable bacteria in the culture was 1.6×10^9 colony-forming units (CFU)/ml. After killing the bacteria by addition of formalin to 0.5% (v/v), the culture was centrifuged at 15,000 × g for 20 minutes at 4 C. The supernatant fluid was passed through a 0.2-μm membrane filter[c] and was designated as CF. The CF was concentrated to 10% of its initial volume by ultrafiltration, using semipermeable membrane.[d] For inoculation, concentrated CF was emulsified in an equal volume of light mineral oil containing a 3% emulsifier,[e] and was used as CF vaccine.

Growth agglutination (GA) test—To determine the agglutinating antibody titer of porcine serum, blood samples were collected from the cranial vena cava of swine before vaccination and at challenge exposure. The GA test[24] was conducted, and titers were expressed as the reciprocal of the number with the highest dilution of serum that had agglutination.

Animals—Six hundred 4-week-old outbred female mice[f] of the ddY strain and eighty 2- to 3-month-old female or castrated male Yorkshire swine[g] were used. Swine were conventionally farrowed and raised in confinement. Swine had GA titers of ≤ 4.

Vaccination and challenge exposure—Mice were inoculated SC at 4 sites in the flank with 0.5 ml of CF vaccine and were randomly allotted into 20 groups of 10 on postvaccination day (PVD) 21. Nonvaccinated control mice were also allotted to 20 groups of 10. Each group was challenge exposed SC in the right inner thigh with 0.1 ml of diluted 24-hour BIB culture (approx 100 LD_{50}) of 1 of 20 strains belonging to 18 serovars and type N (Table 1). The mice were observed each day for 14 days after challenge exposure, and responses were determined by the quantal (live-dead) method.

Swine were inoculated SC twice with 2 and 3 ml of CF vaccine, 3 weeks apart, in the cervical area just caudal to the ear, and were randomly allotted to 20 groups of 2 on the 14th day after the last inoculation of the CF vaccine (PVD 35). Nonvaccinated control swine were also allotted to 20 groups of 2. Each group was challenge exposed intradermally in the flank with 0.1 ml (10^6 or 10^7 CFU) of the BIB culture of 1 of 20 strains. Clinical signs (urticarial lesion, depression, anorexia, difficulty in standing, or death) in swine were observed each day for 14 days after challenge exposure.

Results

Cross protection in vaccinated mice—Control mice died after challenge exposure with all strains. Vaccinated mice survived after challenge exposure with each strain of serovars 1b, 2, 8 (strain Goda), and type N, but mortality occurred in vaccinated mice as follows: 20% to 30% mortality in mice challenge exposed to strains of serovars 1a, 11, 12, 15, 16, or 21; 40% to 50% mortality in mice challenge exposed to serovars 4, 5, 6, 7, or 8 (strain 911); and 60% to 80% mortality in mice challenge exposed to serovars 9, 10, 18, or 19. All vaccinated mice died after challenge exposure with strain 2553 (serovar 20, Table 1).

Cross protection in vaccinated swine—At challenge exposure, the GA titer of serum of vaccinated swine was ≥ 128, whereas nonvaccinated swine retained the titer ≤ 8 (Table 2).

In control groups, 1 of 2 swine challenge exposed to serovar 1a (strain Fujisawa) died in acute phase 4 days after inoculation and another had generalized urticarial lesions with profound depression and anorexia for several days. Swine challenge exposed to serovar 2 (strain Saitama-1) had clinical signs similar to those exposed to serovar 1a; swine challenge exposed to serovar 1b (strain H-12) had somewhat weaker clinical signs than did those challenge exposed to serovars 1a or 2, and swine challenge exposed to serovar 9 (strain 14B) or serovar 10 (strain 2179) developed mild, generalized infection indicated by the presence of 3 to 7 metastatic urticarial lesions for 2

[c] Sartorius, Göttingen, Federal Republic of Germany.
[d] Diaflomembrane type PM 10, Amicon Corp, Lexington, Mass.
[e] Arlacel A, Atlas Powder Co, Wilmington, Del.
[f] Shizuoka Agric Coop Assoc Laboratory Animals, Hamamatsu, Japan.
[g] Minano Agric Coop Assoc Laboratory Animals, Saitama, Japan.

TABLE 2—Clinical responses of swine vaccinated with culture filtrate and nonvaccinated control swine to challenge exposure with *Erysipelothrix rhusiopathiae* strains of various serovars

		Vaccinated				Nonvaccinated			
				Response after challenge exposure				Response after challenge exposure	
Serovar	Strain	Swine No.	GA titer	Erythema	Systemic	Swine No.	GA titer	Erythema*	Systemic
1a	Fujisawa	57	4,096	–	–	56	8	Generalized	+ +
		64	512	–	–	59	4	Generalized	Died
1b	H-12	54	1,024	–	–	55	8	Generalized	–
		61	512	–	–	65	8	Generalized	–
2	Saitama-1	62	1,024	–	–	63	8	Generalized	+ +
		67	2,048	–	–	68	8	Generalized	+
4	2229	69	2,048	–	–	73	4	5 × 6	–
		72	4,096	–	–	79	8	5 × 6	–
5	Pécs 67	74	1,024	–	–	76	4	8 × 11	–
		75	2,048	–	–	77	8	5 × 7	–
6	S-17	24	4,096	–	–	26	8	9 × 10	–
		25	8,192	–	–	27	4	15 × 16	–
7	T-334	76	512	–	–	78	4	10 × 14	–
		77	512	–	–	79	8	10 × 12	–
8	Goda	83	2,048	–	–	85	8	12 × 12	–
		84	4,096	–	–	86	4	12 × 14	–
8	911	28	2,048	–	–	30	4	10 × 13	–
		29	2,048	–	–	31	4	16 × 17	–
9	14B	78	8,192	–	–	80	8	Generalized	–
		81	16,384	–	–	82	8	Generalized	–
10	2179	87	1,024	–	–	89	8	Generalized	–
		88	4,096	–	–	98	8	16 × 18	+
11	IV 12/8	99	1,024	–	–	101	4	17 × 19	–
		100	8,192	–	–	102	4	17 × 17	–
12	Pécs 9	103	4,096	–	–	105	8	9 × 9	–
		104	2,048	–	–	106	4	4 × 6	–
15	Pécs 3597	32	128	–	–	34	4	3.5 × 4.5	–
		33	1,024	–	–	35	4	3 × 3	–
16	T-184	36	512	–	–	38	8	13 × 15	–
		37	512	–	–	39	4	10 × 12	–
18	715	108	256	–	–	110	8	11 × 12	–
		109	256	–	–	111	8	13 × 14	–
19	2017	14	1,024	–	–	16	4	10 × 12	–
		15	512	–	–	17	8	10 × 10	–
20	2553	5	1,024	–	–	7	4	14 × 15	–
		6	512	9 × 10*	–	8	4	10 × 10	–
21	Bâno 36	9	512	–	–	11	4	14 × 14	–
		10	4,096	–	–	13	< 4	4.5 × 5	–
N	S-12	49	1,024	–	–	47	4	10 × 15	–
		50	4,096	–	–	48	4	12 × 16	–

* Maximum size (cm) of erythema at the skin injection site, which was observed for 2 weeks after challenge exposure. + + = Depression, anorexia, and difficulty in standing. + = Temporary depression and anorexia. – = No response. GA = Growth agglutination.

days after exposure. Nonvaccinated swine challenge exposed to the 15 other strains developed local urticarial lesions at the site of intradermal exposure, but had no evidence of generalized infection.

Of 2 vaccinated swine challenge exposed to serovar 20 (strain 2553), 1 developed only a local urticarial lesion. None of the vaccinated swine challenge exposed to 19 other strains belonging to serovars 1a, 1b, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 16, 18, 19, and 21 and type N developed clinical signs of acute erysipelas.

Discussion

Specificity in immunity to challenge exposure with various serovars of *E rhusiopathiae* generally does not occur in swine inoculated with the CF vaccine prepared from attenuated strain Koganei 65-0.15. This observation is based on the apparently complete protection induced in vaccinated swine against challenge exposure with most serovars of pathogenic strains. Therefore, the immunizing antigen in the CF was considered to be cross protective.

In the present experiment, CF vaccine induced a complete protection in swine against challenge exposure with the same serovars 9 (strain 14B) or 10 (strain 2179) as previously used,[14,15] although protection rate in mice was relatively low. The standard adsorbate bacterin[14,15] contained 50% of culture supernatant in addition to bacterial cells. The major difference between those 2 vaccines is of adjuvant, which are aluminum hydroxide gel or light mineral oil with mannide monooleate. Therefore, differences of susceptibility of vaccinated swine to these 2 specific strains are probably caused by quantitative dissimilarities of antigenic stimulation between the 2 vaccines.

In the protection test of mice, CF vaccine induced relatively low protection against strains of serovars 9 (40%), 10 (20%), 18 (20%), or 19 (30%) and no immunity against serovar 20 (strain 2553) in comparison with the previous results[16,17] obtained by live-organism vaccine. This difference may also be attributed to a difference in the degree of antigenic stimulation between CF vaccine and live-organism vaccine. Attenuated *E rhusiopathiae*, strain Koganei 65-0.15, grows extensively in the systemic or-

gans for more than 20 days[h] and causes arthritis in inoculated mice.[18,h] Therefore, vaccinated mice are hyperimmunized by long-lasting systemic infection with the attenuated strain, whereas the antigenic stimulation by a single injection of CF vaccine is limited. However, vaccinated swine did not exhibit clinical signs of acute erysipelas against strains of serovars 9, 10, 18, or 19. Of 2 vaccinated swine, 1 developed only a local urticarial lesion against challenge exposure with serovar 20 (strain 2553). Seemingly, these strains are partially distinct from strain Koganei 65-0.15 (serovar 2) used for the preparation of the CF vaccine, and differences in susceptibility of vaccinated mice and swine to the various strains are probably caused by quantitative (rather than qualitative) antigenic dissimilarities between the vaccine strain and certain challenge strains.

Mice vaccinated with live-organism vaccine prepared from the attenuated strain used in the present study did not die after challenge exposure to *E rhusiopathiae* strains of 20 serovars or one N type, but 30% mortality occurred in vaccinated mice challenge exposed to only serovar 20 (strain 2553).[16,17] In the swine protection test, 1 of 2 vaccinated swine challenge exposed to each of serovars 8 (strain 911) or 20, and 2 of 4 vaccinated swine challenge exposed to each of serovars 9 (strain 14B) or 10 (strain 2179) developed localized urticarial lesions at the site of intradermal exposure, but vaccinated swine challenge exposed to serovars 1a, 1b, 2, 4, 5, 6, 7, 11, 12, 15, 16, 18, 19, or 21 or type N did not have clinical signs of acute erysipelas.[16,17] The immunity stimulated by live cells of attenuated *E rhusiopathiae* is reportedly cell mediated.[25] Mice hyperimmunized against *E rhusiopathiae* had a notable cross protection on challenge exposure with virulent *Listeria monocytogenes*.[26] However, in the present experiment, CF vaccine induced a cross-protection pattern in mice and swine similar to that induced by live-organism vaccine in previous experiments.[16,17] The cross protectivity with CF vaccine in swine was stronger than it was with the live-organism vaccine. Seemingly, cross-protective immunity induced by live-organism vaccine may be caused by specific antigenic stimulation, rather than nonspecific cellular response. Common protective antigen, which is found in CF as a soluble immunizing factor, released from the attenuated live cells by their autolysis as a result of continuous multiplication in the skin injection site of swine,[27] may induce the production of cross-protective antibody. Further investigation on the protective effect of anti-CF serum would be helpful to confirm the important role of CF in cross protection.

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85929/82

FORM 1

COMMONWEALTH OF AUSTRALIA

LODGE AUSTRALIAN 15 JUL 1981 PATENT OFFICE

THE PATENTS ACT 1952-1973

APPLICATION FOR A PATENT

COMPLETE WHERE PROVISIONAL SPECIFICATION No. 85924/82.

We, COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANIZATION, of Limestone Avenue, Campbell, ACT, hereby apply for the grant of a Patent for an invention entitled:

"MASTITIS VACCINE"

which is described in the accompanying provisional specification.

RECEIVED Date 14.7.81 Receipt PE9918 Application — Declaration — Specification 9+TC Drawings —

Our address for service is:

C/- The Director
Bureau of Scientific Services
CSIRO Head Office
Limestone Avenue
CAMPBELL ACT 2601

(Postal address: PO Box 225
DICKSON ACT 2602)

DATED this 14th day of July 1981.

COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANIZATION

By: D Burgess
Registered Patent Attorney

AUSTRALIAN [illegible]

FEE STAMP TO VALUE OF $ 142 ATTACHED

MAIL OFFICER

TO:
THE COMMISSIONER OF PATENTS

(12) AUSTRALIAN PATENT ABSTRACT
(19) AU (11) AU-A-85 929/82

(54) MASTITIS VACCINE

(71) COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANIZATION

(21) 85 929/82 (22) 14.7.81 (23) 12.7.82 (24) 14.7.81
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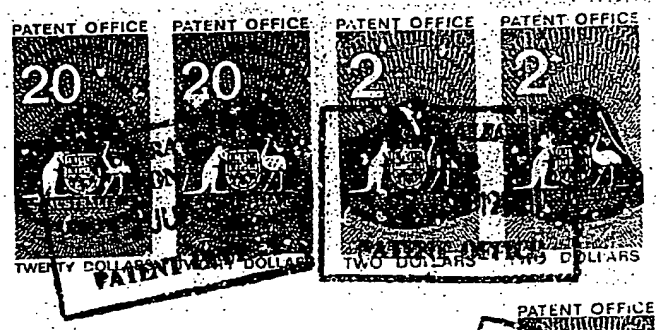
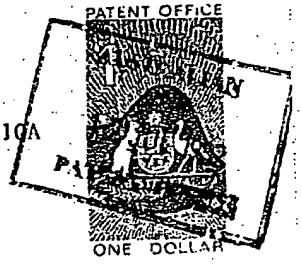
(57) Claim

1. A vaccine effective in the immunisation of ruminants against intermammary challenge by homologous or heterologous strains of S.aureus.

3. A method as claimed in claim 1 in which the strain of S. aureus is a catalase and coagulase - positive strain of ruminant origin.

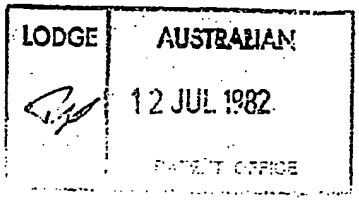




Form 10A

COMMONWEALTH OF AUSTRALIA

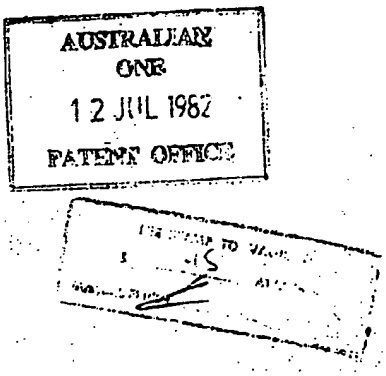
Patents Act 1952



COMPLETE PATENT SPECIFICATION FOR THE INVENTION ENTITLED

"MASTITIS VACCINE"

The following is a full description of this invention, including the best method of performing it known to me:



This invention concerns the immunisation of ruminants against staphlococcal mastitis.

Staphylococcal mastitis is a disease condition affecting approximately 25% of Australian dairy cattle, which is estimated to cost the industry about $30,000,000 each year in lost milk production, culling, antibiotics and costly husbandry procedures.

Traditionally the disease is treated, on the appearance of clinical symptoms, by the infusion of antibiotics which are active against the infective organism, Staphyloccoccus aureus. Such an approach is far from ideal. Apart from being curative rather than preventative, there are economic considerations such as the need for identification and individual treatment of affected animals and the unsuitability of antibiotic-contaminated milk for human consumption or processing. An additional problem is the increasing development of antibiotic-resistant strains of S. aureus. Attention has, therefore, turned towards the possibility of immunizing animals against the infection. Until now, however, there has been no vaccine which will provide resistance to homologous or heterologous strains of S. aureus. The reasons for this are imperfectly understood. However, as we have now developed a technique for preparing a live vaccine which is capable of conferring a useful level of homologous and heterologous immunity, it is thought that previous vaccines, which were dead vaccines, lacked certain essential virulence determinants produced only under in vivo growth conditions.

Accordingly, in its broadest aspect this invention provides a vaccine effective in the immunisation of ruminants

against intramammary challenge by homologous or heterologous strains of S. aureus.

In another aspect the invention provides a method of preparing an effective and safe staphylococcal mastitis vaccine. This method is characterised by multiple passage of a strain of S. aureus until loss of haemolytic activity is noted.

In general terms the vaccine may be prepared by attenuating a catalase and coagulase - positive strain of S. aureus, of ruminant origin, by multiple passage on a suitable bacteriological growth media until loss of haemolytic activity is achieved. The attenuated strain may then be cultured in a suitable bacteriological media, washed free of the media and exotoxins with a non-bacterio-static diluent, and, using the same diluent, diluted to the desired concentration (at least about 10^8 S. aureus/ml.) for administration.

The following is an example of the preparation of an S. aureus vaccine by the method of this invention and of animal trials embracing use of the vaccine:

EXAMPLE

Preparation

A catalase and coagulase positive strain of Staphylococcus aureus (Strain WA 79) was isolated from an acute case of bovine mastitis. It was attentuated by multiple passage on 5% sheep blood agar until haemolytic activity could no longer be observed, after which the strain was maintained on mannitol-salt agar sloped at 4°C. When required, a vaccine was prepared by growing the bacteria in nutrient broth (Oxoi CM1) for 24 hours at 37°C with shaking; the culture

was then washed twice with sterile phosphate-buffered saline (PBS) and adjusted to a concentration of 10^{10}/ml with sterile PBS.

Experimental Procedure

12 pregnant primiparous Merino ewes were maintained in phalaris-white cover pastures with water provided ad libitum. Each ewe was allowed to rear its lamb. The animals were randomly allocated to two treatment groups (Table 1). Ewes in Group 1 were given 1 ml of the vaccine subcutaneously in the left hindleg at 6 weeks and 4 weeks prepartum. Ewes in Group 2 were maintained as a non-vaccinated control.

Serum was prepared from blood samples collected at intervals before and after vaccination. Whey was prepared by centrifugation of milk samples collected from each animal immediately prior to challenge and both serum and whey were stored at -16°C until required for assay.

At 30-35 days post-partum both mammary glands of ewes in both groups were challenged by infusion of 1 ml of viable S. aureus (Strain Wood 46) at a concentration of 10^{6}/ml. Lambs were removed from the ewes for the 6 hours after challenge. Clinical assessments, milk production measurements, leucocyte counts and assays of bacteriological status were made before and at intervals after challenge.

Results

Antibody Responses. Results for titres of anti-staphylococcal agglutinating antibody in blood serum and whey are presented in Table 1. Moderate increases in serum titres were recorded for animals in Group 1, but there was no significant difference

between treatment groups for titres in milk whey immediately prior to challenge.

Opsonisation Assays

The radioisotope technique of Lam & Mathieson (1979 - J.med. Microbiol. 12:459) was used to compare the opsonizing capacity of serum and whey from ewes in each treatment group. The effector cells were neutrophils obtained from involuted mammary glands of multiparous, non-immunised ewes and an incubation period of 30 minutes was allowed. The opsonisation index is computed as equivalent to the percentage of inhibition, due to internalisation, of radioisotope uptake by the cccci. It therefore provides a direct measure of internalisation of cocci by neutrophils. There was no significant difference between the groups for indices for serum collected at the time of the primary and booster vaccinations, however by 5 weeks post-partum the mean index for the serum of animals of Group 1 was significantly greater (62 cf.52) than that for Group 2. There was no significant difference between the opsonisation indices for milk whey at that time.

Clinical Response after Challenge

The numbers of ewes in each group exhibiting clinical signs of acute mastitis are shown in Table 2. In these animals the mammary glands were severely swollen and oedematous; there was depression and pyrexia with rectal temperatures for control ewes reaching 40.4± 0.4°C at 24 hours post challenge.

The proportions of ewes in each group shedding S. aureus in milk in the 48 hours following challenge are also shown in Table 2. It can be seen that there was a marked reduction

in the prcportion of vaccinated ewes which shed the bacteria over this period.

Luecocyte Count

Results for leucocyte counts in milk after challenge are shown in Table 3. Significant leucocytosis developed in all animals, with maximal values occurring 24 hours after infection. However, at 8 days post-challenge the mean value for the control group was significantiy greater than for the vaccinated group.

Milk Production

Data for milk production post-challenge is presented in Fig.1. On day 3 the mean production for ewes of the vaccinated group was significantly better than for the control of ewes and remained so for at least a further 5 days.

The experiments demonstrated that ewes immunised with a vaccine according to this invention developed a considerable degree of immune resistance to challenge with a heterologous strain. On the basis of clinical criteria and changes in milk production, following challenge, the vaccine was considered to provide a useful measure of protection.

Vaccines according to this invention may be administered subcutaneously or intradermally typically (at a concentration of 10^{10}/m) as two doses of 1 ml each at least two weeks apart.

CLAIMS

The claims defining the invention are as follows:

1. A vaccine effective in the immunisation of ruminants against intermammary challenge by homologous or heterologous strains of S.aureus.

2. A method of preparing a vaccine as claimed in claim 1 which comprises the multiple passage of a strain of S. aureus until loss of haemolytic activity is observed.

3. A method as claimed in claim 1 in which the strain of S. aureus is a catalase and coagulase - positive strain of ruminant origin.

4. A method as claimed in either claim 2 or 3 in which, after multiple passage, the attenuated strain is cultured in a suitable bacteriological media, washed free of the media and exotoxins with a non-bacteriostatic diluent and diluted to the desired concentration with the same diluent.

5. A method as claimed in claim 4 in which the desired concentration is at least 10^8 S. aureus/mil.

6. A method of preparing a vaccine as claimed in claim 1, substantially as described in the Example herein.

7. A vaccine as claimed in claim 1 when prepared by the method as claimed in any one of claims 2-6.

Dated this 12th day of July, 1982.

COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANIZATION

TABLE 1

Anti-staphlococcal Agglutination Titres (Log_2) in Blood Serum and Milk They

Treatment Group	Time Before or After Parturition (Weeks)				
	-6 Serum	-4 Serum	0 Serum	+5 Serum	+5 They
1	4.7±0.4	5.7±0.4	7.3±0.3	8.0±0.5	0.4±0.3
2	4.6±0.5	5.2±0.3	3.8±0.2	3.6±0.5	1.2±0.3

Values presented are means ± standard errors

TABLE 2

Proportions of Ewes in Each Treatment Group Exhibiting Clinical Signs of Acute Mastitis and Shedding S. aureus in Milk after Challenge

Treatment Group	Clinical Signs of Acute Mastitis			Shedding S. aureus in Milk		
	Time after challenge (hours)					
	6	24	48	6	24	48
1	1	1	1	7	5	3
2	3	4	4	5	5	4

Total number of animals in Group 1 = 7
Total number of animals in Group 2 = 5

TABLE 3

Leucocyte Counts ($\log_{10}$) per ml before and at intervals after Challenge

Treatment Group	Time after Challenge (hours)				
	0	6	24	48	192
1	5.07±0.05	6.53±0.10	7.16±0.14	6.34±0.17	6.08±0.30
2	4.98±0.15	7.35±0.07	7.82±0.12	7.22±0.35	7.45±0.28

Values are means ± standard errors

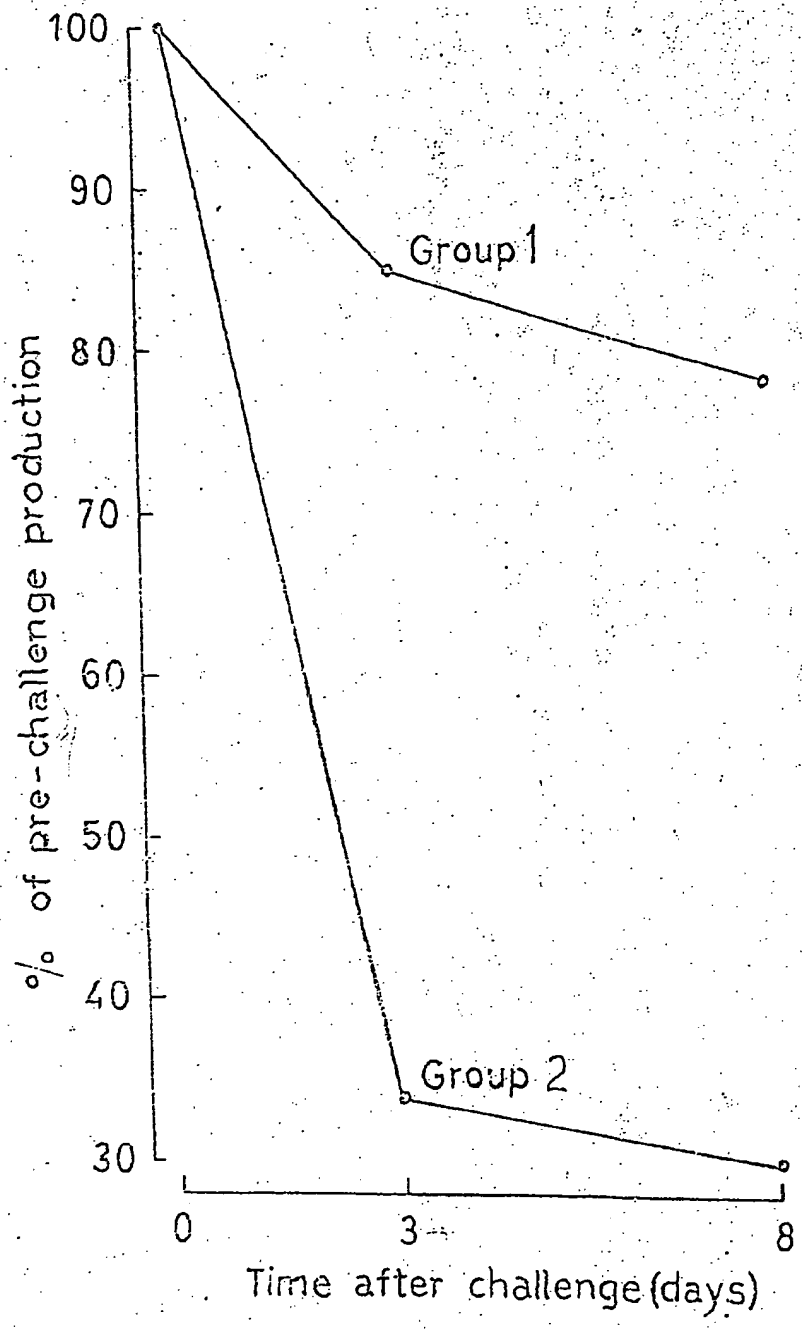


Figure 1. Milk production data for ewes in the 2 treatment groups Values presented are means of post-challenge production expressed as percentages of pre-challenge productions.

THE JOURNAL OF INFECTIOUS DISEASES • VOL. 136, SUPPLEMENT • AUGUST 1977

Temperature-Sensitive Mutants of Type I *Streptococcus pneumoniae*: Preparation, Characterization, and Evidence for Attenuation and Immunogenicity

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From the Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland

Thirteen temperature-sensitive (*ts*) mutants of type I *Streptococcus pneumoniae* were selected after exposure of virulent wild-type (*ts*$^+$) organisms to nitrosoguanidine. Each mutant resembled the *ts*$^+$ parent in properties of α-hemolysis, bile solubility, optochin sensitivity, antibiotic sensitivity, and serotype. Unlike the *ts*$^+$ parent, however, each *ts* mutant was restricted in its capacity to form colonies on blood agar at 38 C. With the exception of two mutants, there was a correlation between the degree of temperature-sensitivity of a mutant and its genetic stability. When inoculated intraperitoneally into mice, 11 of 13 mutants were attenuated and induced homologous resistance. Three mutants (*ts* 1, *ts* 3, and *ts* 4) were also studied in hamsters and were found to be attenuated and immunogenic after intraperitoneal injection. Study of the behavior of mutants *ts* 1, *ts* 3, and *ts* 4 in the blood of hamsters suggested that attenuation may be related, in part, to decreased growth and survival of *ts* organisms at body temperature. Mutants *ts* 1 and *ts* 4 were completely attenuated for hamsters when administered intranasally and induced significant resistance to subsequent challenge with wild-type organisms by the same route. Local administration of *ts* mutants of type I *S. pneumoniae* to hamsters may provide a model for evaluating the potential of live vaccines in the prevention of disease due to bacterial respiratory tract pathogens.

With the recognition of the importance of local immune processes in resistance to viral and mycoplasmal respiratory diseases, emphasis has been placed upon the development of vaccines that stimulate local immunity when introduced into the respiratory passages. One promising approach is based upon the temperature gradient that exists within the respiratory tract. Temperatures range from 27 C in the outer nasal passages to 37 C in the lungs when the environmental temperature is 22 C [1, 2]. To take advantage of this temperature differential, mutants of respiratory tract pathogens have been selected that grow vigorously at 32 C but that do not replicate efficiently at 37 C–38 C in vitro [3–5]. Theoretically, temperature-sensitive (*ts*) mutants of this type should replicate efficiently at temperatures prevalent in the upper respiratory passages and should stimulate local immunity. However, because of genetic restriction on growth at the higher temperature of the lungs, the mutants should not be able to replicate to a titer sufficient to produce disease in the lower respiratory tract.

Because of the encouraging results obtained with *ts* mutants of viral and mycoplasmal respiratory tract pathogens, we wondered whether *ts* mutants of bacterial respiratory pathogens might also merit consideration as potential vaccine candidates. Such an alternate approach to immunoprophylaxis might be considered in the case of certain important bacterial diseases affecting

The authors are grateful to Dr. Gerald Schiffman of the Department of Microbiology and Immunology, State University of New York, Downstate Medical Center, New York, New York, for determinations of antipneumococcal antibodies, and to Drs. Lawrence Senterfit and Sonia Urmacher of the Department of Microbiology, Cornell University Medical Center, New York, New York, for bacteriological and serological characterization of the mutants. Drs. David Alling, Alan Cheever, and Benjamin Prescott of the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, made contributions to this work.

Please address requests for reprints to Dr. Michael B. Grizzard, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014.

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the young, whose immunologic response to some parenterally administered purified polysaccharides is less than optimal [6–8]. These diseases include otitis media due to *Streptococcus pneumoniae* and *Haemophilus influenzae* and meningitis due to *S. pneumoniae*, *H. influenzae*, and group B *Neisseria meningitidis*. *S. pneumoniae*, the major bacterial respiratory tract pathogen of children and adults, was selected to test the feasibility of this approach. This paper summarizes the methods used to prepare and characterize *ts* mutants of type I *S. pneumoniae* and presents evidence of attentuation and immunogenicity of the mutants in experimental animals.

Materials and Methods

Type I S. pneumoniae. The wild-type or *ts*$^+$ strain of type I *S. pneumoniae* employed was isolated in 1935 from the sputum of a patient with lobar pneumonia, was passaged in mice, and was preserved by lyophilization. After reconstitution from the lyophilized state, the strain was passaged five times in mice and 10 times in hamsters [9]. Cloned organisms that were grown in blood agar and that were from the final passage in hamsters were suspended in brain-heart infusion broth with 10% glycerol and stored in 2-ml aliquots at −70 C for subsequent use.

Mutagenesis and selection. A logarithmic-phase culture of *ts*$^+$ *S. pneumoniae* was centrifuged, the pellet was suspended in phosphate-buffered saline (PBS, pH 7.4), and aliquots were dispensed into sterile centrifuge tubes. An appropriate volume of a freshly prepared solution of N-methyl-N'-nitro-N-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, Wis.) was added to half the tubes to bring the concentration of mutagen to 50 μg/ml; an identical volume of PBS was added to the remaining tubes which served as controls. The tubes were then incubated at 37 C, and at 15- to 30-min intervals, bacteria were harvested, washed, and titrated on 5% sheep blood agar plates to obtain a bacterial killing curve. Mutants were selected from nitrosoguanidine-treated suspensions in which the bacterial killing was 10-fold greater than that in the untreated control. Fifty to 100 cfu from nitrosoguanidine-treated suspensions were spread on a series of blood agar plates that were incubated at 32 C for 24–48 hr and then used as templates for subsequent replica plating [10]. Two blood agar replicates of each template were prepared; one was incubated at 32 C (a permissive temperature) and the other at 38 C (a restrictive temperature). Colonies appearing on the 32 C plate without a counterpart on the 38 C plate were picked, streaked on blood agar, and tested again at the permissive and restrictive temperatures. Putative *ts* mutants were then cloned four times. Organisms grown in blood agar that were from the final cloning were frozen in brain-heart infusion broth with 10% glycerol.

Efficiency of colony formation. Serial 10-fold dilutions of suspensions of *ts*$^+$ and of *ts S. pneumoniae* were spread on blood agar plates that were incubated at 32 C, 34 C, 36 C, 37 C, 38 C, or 39 C. Colonies were counted after incubation for 48 hr.

In vitro genetic stability. The genetic stability of *ts* mutants of type I *S. pneumoniae* was examined on agar and in liquid media. The frequency of reversion on 5% sheep blood agar was estimated from the ratio of colony counts obtained after incubation for 48 hr at 32 C and 38 C (cfu_{38}/cfu_{32}). Clonal analysis indicated that colonies which developed at 38 C contained organisms that subsequently produced colonies with high efficiency at the restrictive temperature ($cfu_{38}/cfu_{32} = 1$); such organisms were therefore considered to be revertants. Reversion frequencies in trypticase soy and brain-heart infusion broths were estimated by tube dilution tests. Serial decimal dilutions of *ts* organisms were prepared in broth and incubated for 24 hr at 38 C. The reversion frequency was the smallest inoculum of *ts* organisms that induced development of turbidity and/or growth of pneumococci capable of colony formation on blood agar at 38 C.

Studies in mice. Groups of eight to 10 Swiss mice were inoculated ip with 0.2-ml volumes of decimal dilutions of pneumococcal suspension. Control mice were inoculated with heat-killed organisms (56 C for 2 hr) or broth alone. The LD_{50} was calculated on the basis of the cumulative mortality rate during the week after inoculation [11]. Mice inoculated one month previously with one of the *ts* mutants or broth were challenged ip with 200 LD_{50} of virulent *ts*$^+$ organisms,

and deaths were recorded for seven days afterwards.

Studies in hamsters. Male golden Syrian hamsters (80 g) were obtained from Charles River-Lakeview, Newfield, N.J. On day 0, hamsters were inoculated either ip or intranasally with 10^7 cfu of *ts*$^+$ or *ts S. pneumoniae* suspended in 0.2 ml of brain-heart infusion broth. There were 54 animals in each experimental group. Control animals were inoculated with broth or heat-killed organisms. Morbidity and mortality in control and experimental groups were recorded daily for one week after inoculation. On days 0, 1, 2, 3, 4, 5, and 7 after inoculation, three animals in each experimental group were exsanguinated. Sheep blood agar plates were immediately inoculated with decimal dilutions of unclotted hamster blood and incubated at 32 C for 48 hr; colony counts were used to quantitate pneumococcal bacteremia. Heart, lung, and peritoneal tissues were removed and fixed and stained for histological analysis. Antibodies to *S. pneumoniae* in serum were measured by a sensitive radioimmunoassay system [12].

Ten weeks after primary inoculation, hamsters were challenged intranasally with 10^7 cfu of *ts*$^+$ *S. pneumoniae* to determine whether homologous resistance had developed. Morbidity and mortality among experimental and control groups were monitored for two weeks after challenge.

Results

General properties of ts mutants of S. pneumoniae. A total of 13 *ts* mutants of type I *S. pneumoniae* were selected in two separate mutagenesis experiments. The *ts* mutants were phenotypically similar to the wild-type parent in properties of α-hemolysis, bile solubility, and optochin sensitivity [9]. The mean bactericidal levels of penicillin and erythromycin for *ts* organisms were essentially identical to those for *ts*$^+$ organisms [9]. In addition, each of the mutants retained type I immunological properties, as determined by agglutination and Quellung tests [9]. Capsular polysaccharides derived from the *ts* mutants were immunochemically similar to each other and to those of the *ts*$^+$ parent, as indicated by immunodiffusion analysis and by competitive antigen binding [9].

Efficiency of colony formation and genetic stability of ts mutants of S. pneumoniae. The efficiency of colony formation by three representative mutants on blood agar at several different incubation temperatures is compared with that of the *ts*$^+$ parent in figure 1. The shutoff temperature was defined as the lowest temperature at which a ≧1,000-fold reduction in colony-forming capacity occurred. Whereas colony formation by *ts*$^+$ organisms was essentially unaffected by a temperature of as high as 39 C, the three *ts* mutants exhibited a spectrum of temperature sensitivity: *ts* 1 shut off at 38 C, *ts* 4 at 37 C, and *ts* 3 at 36 C.

The relation between the temperature sensitivity of a mutant (i.e., shutoff temperature) and in vitro genetic stability is shown in figure 2. It should be noted that detection of revertants was dependent in part upon the medium employed. Mutants *ts* 2, *ts* 9, and *ts* 7, which were the least temperature-sensitive (shutoff temperature,

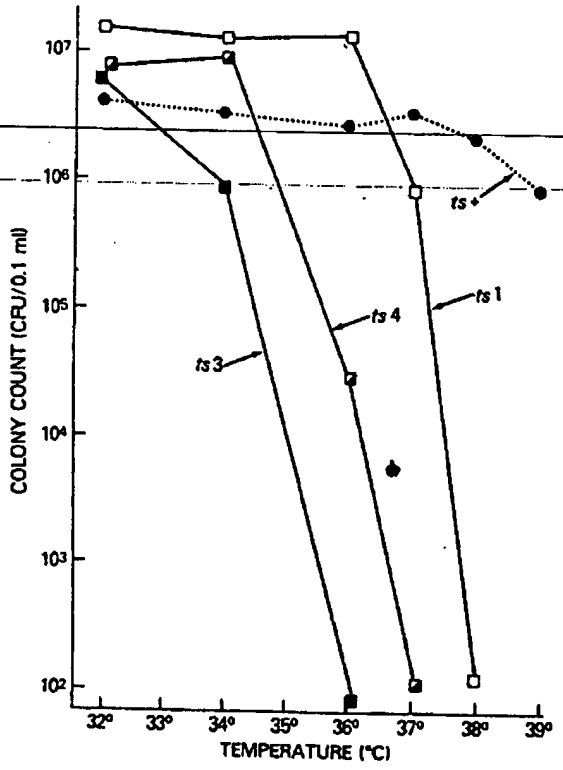

Figure 1. Efficiency of colony formation by temperature-sensitive (*ts*) and wild-type (*ts*$^+$) type I *Streptococcus pneumoniae* on 5% sheep blood agar at different temperatures.

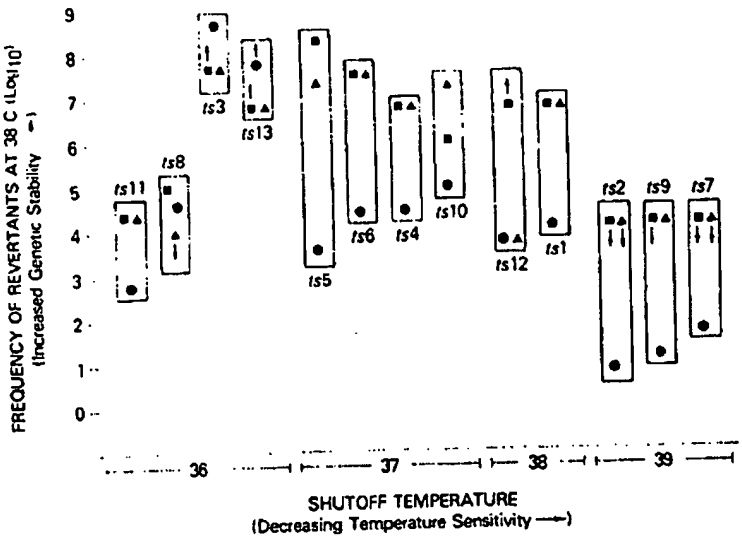

Figure 2. Relation of shutoff temperature of temperature-sensitive (*ts*) mutants of type I *Streptococcus pneumoniae* to in vitro genetic stability. Detection of revertants was in part dependent upon the medium employed: (●) = 5% sheep blood agar; (■) = trypticase soy broth; (▲) = brain-heart infusion broth.

39 C), were also the least stable. Mutants *ts* 1, *ts* 4, *ts* 5, *ts* 6, *ts* 10, and *ts* 12, which were of intermediate temperature sensitivity (shutoff temperature, 37 C–38 C), were of intermediate genetic stability. Mutants *ts* 3 and *ts* 13, which were among the most temperature-sensitive of the mutants (shutoff temperature, 36 C), were also the most stable genetically. Mutants *ts* 11 and *ts* 8, which were also among the most temperature-sensitive of the mutants, had intermediate genetic stability and thus were apparent exceptions to the general correlation between temperature sensitivity and genetic stability.

Virulence and immunogenicity of ts mutants of S. pneumoniae in mice. The virulence of *ts*⁺ and *ts* type I *S. pneumoniae* administered ip to mice was assessed as shown in figure 3. The *ts*⁺ parent, with a shutoff temperature of >39 C, was highly lethal with an LD_{50} of $10^{0.2}$ cfu. Significantly, 11 of the 13 *ts* mutants were markedly attenuated; these attenuated mutants had an LD_{50} that ranged from 10^5 to 10^7 cfu. There was no clear-cut relation between shutoff temperature and virulence.

Mice inoculated one month previously with 10^4—10^7 cfu of one of the 11 attenuated *ts* mutants were challenged ip with a lethal dose of *ts*⁺ organisms to determine whether resistance had been induced. The number of mice that were challenged in each group ranged from 11 to 66. As shown in figure 4, 24%–100% of mice previously inoculated with a *ts* mutant survived a

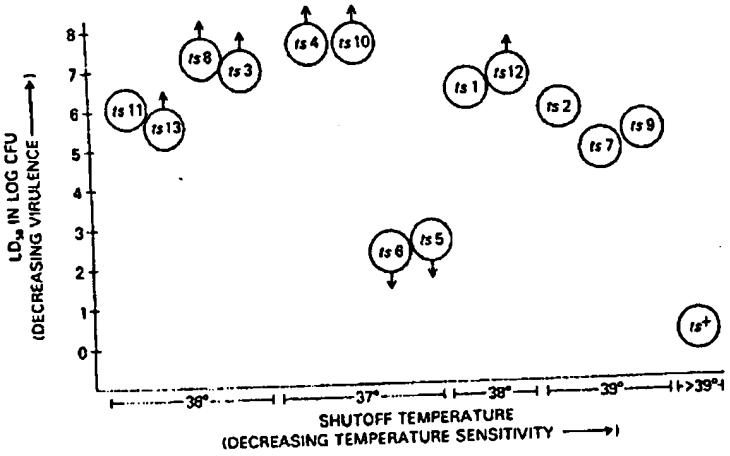

Figure 3. Virulence of temperature-sensitive (*ts*) mutants of type I *Streptococcus pneumoniae* administered ip to mice. (↑) = no mortality at highest dose tested; (↓) = no survival at lowest dose tested.

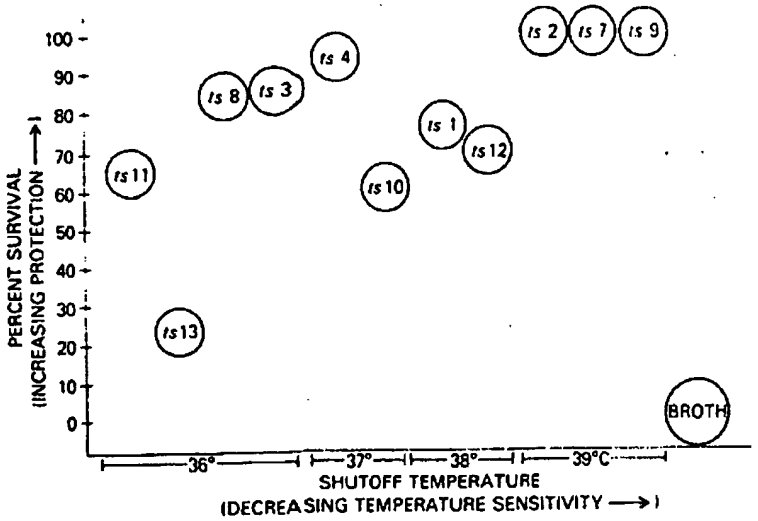

Figure 4. Resistance of mice inoculated ip four weeks previously with temperature-sensitive (*ts*) mutants of type I *Streptococcus pneumoniae* to lethal ip challenge with 200 LD_{50} of the wild-type (ts^+) organisms. All groups given *ts* mutants were significantly protected ($P < 0.001$, χ^2 analysis) as compared with broth-inoculated controls.

challenge with 200 LD_{50} of ts^+ organisms, whereas all of the control animals inoculated with broth died ($P < 0.001$, χ^2 analysis). Although there was no consistent relation between the shutoff temperature of a mutant and the protective effect induced in the mouse, mice that survived infection with mutants with a shutoff temperature of 39 C were uniformly resistant, whereas mutants with a lower shutoff temperature were more variable in their protective effect.

The relation between the residual virulence and immunogenicity associated with different doses of three selected *ts* mutants is shown in figure 5. The ts^+ parent was highly lethal, with 1

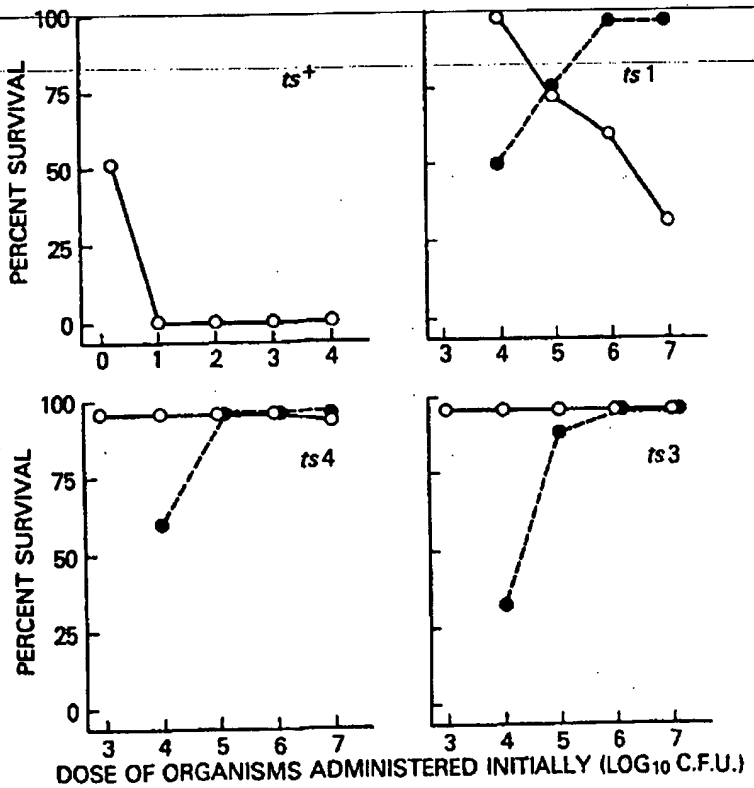

Figure 5. Response of mice to ip inoculation with temperature-sensitive (*ts*) or wild-type (ts^+) type I *Streptococcus pneumoniae* and to subsequent lethal ip challenge with ts^+ organisms. (o——o) = survival after primary inoculation; (●- - - -●) = survival after challenge with 200 LD_{50} of ts^+ organisms.

cfu producing a mortality rate of 50%. Mutant *ts* 1 produced significant dose-dependent mortality in inocula ranging from 10^5 to 10^7 cfu, but this effect was less than that seen with the wild-type parent. Inoculation of as many as 10^7 cfu of *ts* 4 or *ts* 3 organisms failed to induce significant mortality. Surviving mice were challenged ip one month later with 200 LD_{50} of *ts*$^+$ organisms. The dose of *ts* 1 organisms that induced complete resistance also produced significant mortality. In contrast, the dose of *ts* 4 and *ts* 3 organisms that induced complete or almost complete resistance to challenge with *ts*$^+$ organisms did not kill mice. Thus, there appeared to be a significant "safety factor" inherent in these two mutants.

Virulence and immunogenicity of ts mutants of S. pneumoniae in hamsters. Evidence of attenuation and immunogenicity of mutants *ts* 1, *ts* 4, and *ts* 3 was sought with use of another animal species, the golden Syrian hamster. Before inoculation, the mean rectal temperature of 10 lightly anesthetized hamsters was found to be 37.4 C (range, 36.2 C–38.3 C). It appeared that the extent and duration of bacteremia after ip inoculation of 10^7 cfu of *ts*$^+$ or *ts* organisms into hamsters was related to the shutoff temperature of the organism administered (figure 6). Thus, *ts*$^+$ *S. pneumoniae*, which shut off at a temperature of ≥1.6 C above the mean rectal temperature of the hamster, grew readily in the blood, attained peak concentrations in about 48 hr, and persisted for five days. Mutant *ts* 1, which shut off at 0.6 C above the hamster rectal temperature, failed to show an increase in titer in the blood but persisted at a detectable level for four days. Mutants *ts* 4 and *ts* 3, which shut off at 0.4 C and 1.4 C, respectively, below the hamster rectal temperature, failed to show an increase in titer and were detected only rarely, and then in low titer, after inoculation. Clearance of circulating organisms, whether *ts*$^+$ or *ts*, was associated with the appearance of type-specific antibodies by the third to fourth day, as detected by radioimmunoassay.

The degree of illness and histological evidence of disease observed after ip inoculation of 10^7 cfu of *ts*$^+$ or *ts* organisms paralleled the degree of bacteremia observed (table 1). Wild-type organisms reached levels of 10^8 cfu/ml in blood at 48 hr, and 90% of inoculated animals died. The rate of morbidity was 100%; 79% of the animals developed pericarditis, and 88% had peritonitis. Mutant *ts* 1 produced a low level of bacteremia, less morbidity, and no mortality. Finally, mutants *ts* 4 and *ts* 3, which were rapidly cleared from the blood, failed to kill and produced very little morbidity.

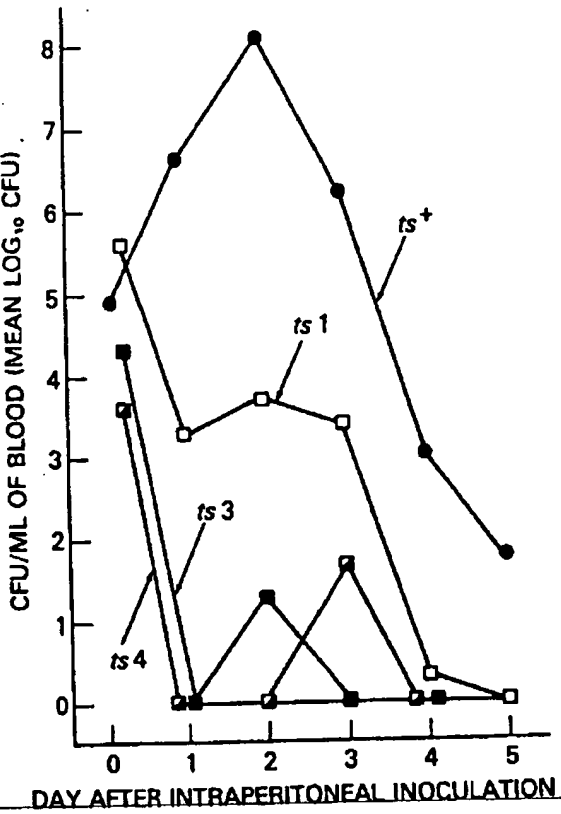

Figure 6. Bacteremia in hamsters after ip inoculation of 10^7 cfu of temperature-sensitive (*ts*) or wild-type (*ts*$^+$) type I *Streptococcus pneumoniae*.

Attenuation of the *ts* mutants was more striking when organisms were administered intranasally. Wild-type organisms, which reached levels of 10^7 cfu/ml in blood by 48 hr after inoculation, killed 60% of the inoculated animals, and those that did not die became ill; 38% of the hamsters developed pericarditis, and 33% had peritonitis. Unlike the *ts*$^+$ parent, each of the three *ts* mutants failed to produce bacteremia or disease and failed to stimulate the production of serum antibody by day 7.

Hamsters inoculated 10 weeks previously either ip or intranasally with 10^7 cfu of *ts S. pneumoniae* were challenged intranasally with 10^7 cfu of *ts*$^+$ organisms (table 2). Animals that were

Table 1. Response of hamsters to inoculation with 10^7 cfu of temperature-sensitive (*ts*) or wild-type (ts^+) type 1 *Streptococcus pneumoniae*.

Route of administration, organism	Bacteremia at 48 hr (mean log10 cfu/ml)	Mortality rate at one week (%)*	Morbidity rate at one week (%)*	Percentage of animals with Pericarditis during first week†	Percentage of animals with Peritonitis during first week†	Serum antibody response by day 7‡
Intraperitoneal						
ts^+	8.1	90	100	79	88	Yes
ts 1	3.8	0	64	14	33	Yes
ts 4	1.0	0	5	6	28	Yes
ts 3	1.3	0	0	0	11	Yes
Heat-killed §	None	0	0	5	0	Yes
Intranasal						
ts^+	7.4	60	100	38	33	Yes
ts 1	1.0	0	0	0	0	No
ts 4	1.0	0	0	0	0	No
ts 3	1.0	0	0	0	0	No
Heat-killed §	None	0	0	0	0	No

*Each group included 30–39 hamsters.

†Tissues were taken at daily intervals over seven days from a total of 13–21 animals in each group and examined histologically. The value reported is the cumulative percentage.

‡As measured by radioimmunoassay.

§Included ts^+, *ts* 1, *ts* 4, and *ts* 3 organisms.

previously inoculated ip with *ts* 1, *ts* 3, *ts* 4, or heat-killed organisms exhibited significant resistance to intranasal challenge with virulent organisms. Although hamsters failed to develop detectable serum antibody one week after intranasal administration of *ts* 1 or *ts* 4 organisms, such animals were demonstrably resistant to intranasal challenge with ts^+ organisms 10 weeks later. This finding suggests that the *ts* 1 and *ts* 4 mutants were able to infect when instilled locally into the respiratory tract and that this type of infection induced immunity. Animals that received living *ts* 3 organisms or heat-killed organisms (ts^+, *ts* 1, *ts* 4, or *ts* 3) intranasally were not resistant to subsequent intranasal challenge with ts^+ organisms.

Discussion

That the evaluation of *ts* bacterial mutants as a possible source of vaccines to prevent bacterial disease has been limited is surprising considering the extensive study of such mutants in molecular genetics. A temperature-sensitive filamentous mutant of *Salmonella enteritidis*, studied extensively in mice and rats, was found to be attenuated and to afford substantial protection against oral infection with *S. enteritidis* [13].

Effective polysaccharide vaccines for the prevention of pneumococcal pneumonia in adults have been developed [14]. The use of such vaccines in prevention of pneumococcal disease in infants and young children appears problematic, however, since serologic response in this age group to some parenterally administered polysaccharides is poor [6–8, 14]. Thus, live attenuated mutants of *S. pneumoniae* administered locally might provide an alternate approach to the immunoprophylaxis of pediatric pneumococcal disease, which occurs when immunologic responsiveness to polysaccharide vaccines is less than optimal.

In the present study *ts* mutants of type I *S. pneumoniae* were selected after treatment of virulent organisms with nitrosoguanidine. The mutants resembled the ts^+ parent in bacteriological and immunochemical properties and, more important, retained sensitivity to antibiotics. The *ts* mutants differed from the ts^+ parent, however, in that each of the mutants grew poorly or not at all at temperatures near that of the body core of humans, a characteristic that theoretically should restrict systemic growth. However, growth of the mutants occurred in vitro at temperatures comparable to those in the upper airways.

It is important that infection initiated by *ts*

Table 2. Response of hamsters inoculated 10 weeks previously with 10^7 cfu of temperature-sensitive (*ts*) type I *Streptococcus pneumoniae* to intranasal challenge with 10^7 cfu of wild-type (ts^+) organisms.

Route of primary inoculation, material inoculated (no. challenged)	Mortality rate at one week (%)	Morbidity rate during first week (%)
Intraperitoneal and intranasal, broth (40)	45	98
Intraperitoneal		
ts 1 (21)	0*	0*
ts 4 (23)	4*	4*
ts 3 (24)	0*	4*
Heat-killed (32)†	16*	41*
Intranasal		
ts 1 (21)	14*‡	14*‡
ts 4 (22)	9*‡	9*‡
ts 3 (21)	57	100
Heat-killed (28)†	60	96

*These rates were significantly less than the corresponding rates in broth-inoculated controls ($P < 0.05$, Fisher's exact test).

†Included ts^+, *ts* 1, *ts* 4, and *ts* 3 organisms.

‡These rates were significantly less than the corresponding rates for animals receiving heat-killed organisms intranasally ($P < 0.05$, Fisher's exact test).

mutants be sufficiently extensive to stimulate local and systemic immunity, yet be restricted enough to be clinically inapparent. Most of the *ts* mutants of type I *S. pneumoniae* examined in the mouse and the hamster appeared to meet these criteria. Of particular interest was the observation that mutants *ts* 1 and *ts* 4 were attenuated and protective when inoculated locally into the respiratory tract of hamsters. These encouraging results suggest that local administration of *ts* mutants of type I *S. pneumoniae* to hamsters may provide a model for evaluating the potential of live vaccine for the prevention of disease due to bacterial respiratory tract pathogens.

The question of the importance of the *ts* lesion or lesions in attenuation remains unanswered at this time; however, the relation of temperature sensitivity to the extent of bacteremia in a limited test of three mutants suggests that this property may be of significance in attenuation. It is very likely that attenuation is a composite property that is contributed to by mutagen-induced non-*ts* as well as *ts* mutations.

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SHORT COMMUNICATIONS

Immunization of Mice Against *Streptococcus suis* Serotype 2 Infections using a Live Avirulent Strain

Sylvain Quessy, J. Daniel Dubreuil and Robert Higgins

ABSTRACT

In this study, the IgG response of mice injected with two virulent strains and one avirulent *Streptococcus suis* capsular type 2 strain was compared by Western blotting. The serum from mice immunized against the avirulent strain could recognize most proteins of the various strains tested and similar results were obtained with serum from mice injected with virulent strains. The live avirulent strain was injected twice (days 0 and 10) to groups of five mice, and four virulent strains from different geographical origins were used to challenge the animals. All mice, except one in one group, survived the challenge. These results suggest that a live avirulent strain could be used for immunization of swine, the natural host.

RÉSUMÉ

Dans cette étude, la technique d'immunobuvardage a été utilisée pour comparer la réponse en IgG de souris auxquelles on a administré une souche avirulente de *Streptococcus suis* serotype 2 à celle de souris auxquelles on a administré deux souches virulentes du même sérotype. Le sérum des souris immunisées avec la souche avirulente a reconnu la plupart des protéines qui étaient reconnues par le sérum des souris immunisées avec les souches virulentes. La souche avirulente a été injectée aux jours 0 et 10 à des groupes de cinq souris. Les souris furent ensuite infectées en utilisant, pour les différents groupes, une des quatres souches virulentes provenant de différentes régions géographiques. Toutes les souris, sauf une dans un groupe, furent protégées contre l'infection. Ces résultats suggèrent que l'utilisation de cette souche avirulente devrait être considérée pour d'éventuels essais de protection chez l'hôte naturel, le porc, en utilisant la bactérie vivante.

Streptococcus suis capsular type 2 is an important swine pathogen, causing mainly meningitis, septicemia and arthritis (1). Attempts to control diseases with antibiotics and/or vaccination have often been disappointing, even if autogenous inactivated whole cell vaccines have shown promise (1). Successful passive immunization of mice using antisera directed against different *S. suis* proteins have been reported (2,3) but active immunization against *S. suis* cellular proteins of a given strain failed to protect mice against heterologous strains (4). Live *S. suis* strains have previously been used to protect pigs against the disease but several injections were necessary for good protection (5).

In a previous study, it was noted that the electrophoretic protein profile of an avirulent strain was similar to those of virulent isolates (6). The failure of some avirulent strains to cause disease may be related, at least in part, to their inability to increase capsule production *in vivo* (6). One aim of this study was to compare the IgG response of mice immunized with an avirulent strain and virulent strains of *S. suis*. Another objective was to evaluate the protective potential of this avirulent isolate using a murine experimental model of infection (7). This model has been used in pathological studies (8) and in attempts to predict virulence for the natural host (9).

Five *S. suis* capsular type 2 strains were used; the reference strain (735), isolated in Denmark, was provided by Dr. J. Henrichsen, Statens Seruminstitut, Copenhagen. One isolate from the United States, AAH4, was provided by Dr. Brad Fenwick, Kansas State University. One Mexican isolate, J590, was provided by Dr. Jose Luis Monter Flores, University of Toluca. Two isolates, 1591 and 1330, were from our collection. Strain 1330 was avirulent whereas the other four strains were virulent for mice and pigs (7).

We evaluated, by Western blotting, the ability of IgG produced against various strains to recognize proteins from homologous and heterologous strains. Sera of mice injected with 10^8 cells (formalin-killed) of strains 1330, 735 and 1591 were used. For Western blots, cells were cultured overnight in Todd-Hewitt broth at 37°C, harvested by centrifugation, washed and resuspended in 3 mL of K_2HPO_4 (0.1M, pH 7.0). Cells were then processed three times in a French press cell, treated with lysozyme (5 mg/mL), and the supernatants, containing cytoplasmic and membrane proteins, were recovered after centrifugation (12,000 × *g*, 20 min). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% acrylamide) was then performed (10) in order to separate cellular proteins. Following SDS-PAGE, material was transferred from the slab gel to the nitrocellulose

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FIGURE 1

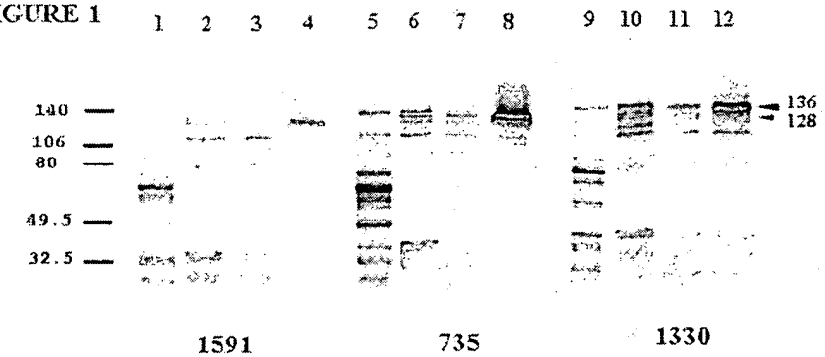

Fig. 1. Western blots of cellular proteins of *Streptococcus suis* capsular type 2 strains (7.5% acrylamide-SDS-PAGE). Protein profiles were revealed using mice antisera against strain 1330 (lanes 2, 6,10), strain 1591 (lanes 3, 7, 11) and strain 735 (4, 8, 12). Molecular weight markers in kDa. Below, strain identification numbers. Lanes 1, 5 and 9 were Coomassie blue stained proteins on acrylamide gels before transfer to nitrocellulose.

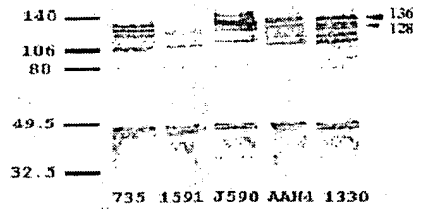

Fig. 2. Comparison, by Western blots, of cellular proteins of various *Streptococcus suis* capsular type 2 strains (7.5% acrylamide-SDS-PAGE) using mice antisera against strain 1330. Molecular weight markers in kDa. Below, strain identification numbers.

membrane by the methanol-Tris-glycine system (11) and the protein profiles were revealed using the various antisera and a peroxidase-labeled goat antiserum raised against murine IgG (Sigma Chemicals, St.Louis, Missouri) and 4-chloro-1-naphthol in cold methanol mixed with H_2O_2.

For immunization assays, four groups of five mice were injected intraperitoneally with 10^7 live cells of strain 1330 on days 0 and 14. Four other control groups were injected with PBS only. All groups of mice were challenged on day 21 by intraperitoneal injection of 10^8 cells of one of the four virulent strains. Western blots were repeated three times, the experiments with animals were repeated twice and the guidelines of the Guide to the Care and Use of Experimental Animals from the Canadian Council on Animal Care were followed.

The antisera raised against the various strains recognized most proteins of these strains and from two other virulent strains. However, some differences were detected in each strain using the different antisera. Mouse antisera against the avirulent strain recognized most proteins also recognized by antisera against the virulent strains (Fig. 1). This indicated that the mouse IgG response to the avirulent strain could recognize many proteins of virulent strains. In particular, the antiserum obtained by injecting mice with the avirulent strain recognized a protein of about 136 kDa in all virulent strains except one; this protein was not dectected in strain 1591 as previously noted (4,9), but was present in strain 1330 (Fig. 2). This 136 kDa protein was shown to be the most immunogenic *S. suis* cellular protein (4), and was recognized by a monoclonal antibody raised against the muraminidase released protein (MRP) (9,12).

Since the avirulent strain led to the production of IgG recognizing many proteins of virulent isolates and since antibodies directed against some proteinaceous epitopes had been shown to protect against the disease (4), it was suggested that antibodies directed against the avirulent strain proteins could induce protection against the virulent strains. Indeed, all mice from three groups were protected against mortality while four of five mice were protected in the fourth group (J590) (Table I). All mice, except one, died in the control groups.

Considerable genetic diversity has been found among *S. suis* isolates (13), but the avirulent strain succeeded in inducing a protection in mice against virulent strains from various geographical origins. Since a 110 kDa extracellular factor, previously reported as a virulence marker (12), was not detected in this strain it could indicate that this factor is not essential for protection.

In a previous study, the 136 kDa cellular protein was recognized by antibodies produced against a 128 kDa cellular protein, present in all strains (Fig. 1)(9). Since the sera of mice immunized with strain 1591, that does not possess the 136 kDa protein, recognized a 136 kDa in all other tested strains, it could also indicate that this strain possesses a protein, probably the 128 kDa protein, with epitopes shared by the 136 kDa protein.

Other structures, not considered in this study, such as capsular polysaccharides, may be involved in the immunity against *S. suis* but *S. suis* polysaccharides are poorly immunogenic (14). The IgG response of mice and pigs to *S. suis* capsular type 2 cellular proteins was shown to be similar (4). Thus, this study suggests that the avirulent strain 1330 would be a good candidate for vaccination of swine with live bacteria.

TABLE I. Active immunization of mice against virulent strains of *Streptococcus suis* serotype 2 using a live avirulent strain

Mice challenged[a] with *S. suis* strain	No. of sick mice[b]/ No. of injected mice (Control groups[c])	No. of dead mice/ No. of injected mice (Control groups)
735	0/5 (5/5)	0/5 (4/5)
J 590	1/5 (5/5)	1/5 (5/5)
1591	1/5 (5/5)	0/5 (5/5)
AAH4	0/5 (5/5)	0/5 (5/5)
1330	(0/5)	(0/5)

[a] Mice were injected intraperitoneally with 10^8 CFU of each strain one week following the second injection with strain 1330

[b] Mean numbers of mice which showed nervous signs and/or prostration during the week following the experiment. Results are the mean of two separate experiments

[c] Control groups were injected twice with PBS before the challenge

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Immunization of Pigs Against *Streptococcus suis* Serotype 2 Infection Using a Live Avirulent Strain

Philippe Busque, Robert Higgins, François Caya, and Sylvain Quessy

ABSTRACT

Sreptococcus suis capsular type 2 is still an important cause of economic losses in the swine industry. At the present time, vaccination of pigs against this infection is generally carried out with autogenous bacterins and results are equivocal. In this study, the protective effect of a live avirulent *S. suis* type 2 strain (#1330) which had induced a good protection in mice, was evaluated in swine. The experiment was performed in triplicate using 4 week-old piglets. A total of 15 piglets were vaccinated 3 times, 15 others were vaccinated 2 times, and 15 piglets were injected 3 times with sterile Todd-Hewitt broth. Using an indirect ELISA, an increase in the IgG response to *S. suis* antigens was noted in 27 of the 30 vaccinated piglets. On day 21 post-vaccination, all animals were challenged intravenously with a virulent *S. suis* type 2 strain (#999). In the 2 vaccinated groups, 26 animals were fully protected. Only 1 out of the 15 piglets vaccinated 3 times developed mild clinical signs. In the group vaccinated twice, 3 piglets showed clinical signs and 1 of them died after the challenge. In the control group, 7 animals died out of the 11 with clinical signs of infection. In conclusion, a protective immunity was observed in swine when using strain 1330. However, more studies are needed to assess the use of a live *S. suis* strain in a vaccine for pigs.

RÉSUMÉ

Les infections dues à *Sreptococcus suis* sérotype 2 sont toujours une cause importante de pertes économiques pour l'industrie porcine. Jusqu'à maintenant, la majorité des essais de vaccination ont été effectués avec des bactérines autogènes et les résultats sont très mitigés. Cette étude avait pour objectif d'évaluer la capacité d'une souche vivante et avirulente de *S. suis* type 2 (#1330) d'induire une protection chez le porc. Cette souche souche avait auparavant induit une protection chez la souris. Dans la présente étude, comportant trois expériences répétées avec des porcelets âgés de quatre semaines, un nombre total de 15 porcelets ont été vaccinés trois fois, 15 autres ont été vaccinés deux fois et 15 porcelets ont reçu trois injections du bouillon de culture stérile Todd-Hewitt. À l'aide d'un test ELISA indirect, une augmentation du titre d'anticorps contre les antigènes de *S. suis* a été notée chez 27 des 30 porcelets vaccinés. Au jour 21 post-vaccination, tous les animaux ont reçu, par voie intra-veineuse, une injection de défi avec une souche virulente de *S. suis* sérotype 2 (#999). Dans les deux groupes d'animaux vaccinés, 26 porcelets sur 30 ont été protégés complètement. Parmi ceux ayant reçu trois doses du vaccin, un seul porcelet a manifesté des signes cliniques. Dans le groupe d'animaux vaccinés deux fois, trois porcelets ont présenté des signes cliniques et l'un d'eux est mort après l'injection de défi. Dans le groupe des témoins, 11 animaux ont présenté des signes cliniques et sept d'entre eux sont morts. En conclusion, une immunité protectrice a été observée chez l'espèce porcine lors de l'utilisation de la souche 1330 comme vaccin. Toutefois, d'autres études sont nécessaires avant de permettre l'utilisation d'une souche vivante de *S. suis* comme vaccin.

INTRODUCTION

Streptococcus suis is an important pathogen of swine causing mainly septicemia, meningitis and endocarditis. Serotype 2 is the most common capsular type recovered from cases of meningitis in weaned pigs in the United Kingdom, North America and the Netherlands (1–3). It is also associated with various types of infections in different animal species, as well as in humans (4,5). Economic losses due to *S. suis* are important and conventional control measures, such as vaccination, have so far given unsatisfactory results (6). Holt et al (7) found that numerous repeated vaccinations with inactivated cells were needed to induce a good protection. Injections of purified capsular polysaccharides in pigs failed to induce adequate protection (8) and poorly encapsulated strains appeared to be as immunogenic as fully encapsulated ones (9). However, passive and active immunization using different cell wall proteins succeeded in protecting mice against the infection (10–12). A few *S. suis* capsular type 2 strains were recently shown to be avirulent in pigs as well as in the mouse model of infection (13). One of them, strain 1330, harbored a highly immunogenic 135 kDa protein which is also present in virulent isolates. It succeeded in inducing a complete protection in mice against the experimental infection with virulent strains (14). The purpose of this study was to monitor the protective effect of vaccination with *S. suis* avirulent strain 1330 and

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TABLE I. Evaluation of antibody response and protection induced by vaccination of piglets with the live avirulent *Streptococcus suis* serotype 2 strain 1330

Experiment	Vaccination protocol	Mean of titer increase[a]	No. of sick pigs[b]/ No. of challenged pigs	No. of dead pigs[c]/ No. of challenged pigs	No. of pigs with *S. suis* in tissues[d]/ No. of challenged pigs
1	3 doses	5.4	0/5	0/5	0/5
	2 doses	3.5	0/5	0/5	0/5
	control	0	4/5	4/5	4/5
2	3 doses	8.5	0/5	0/5	0/5
	2 doses	2.5	2/5	1/5	1/5
	control	0	4/5	2/5	3/5
3	3 doses	8.8	1/5	0/5	0/5
	2 doses	1.8	1/5	0/5	0/5
	control	0	3/5	2/5	2/5

[a] Value represents mean antibody titer increase of animals from 1 group
[b] Nervous signs, lameness, and decubitus for more than 12 h were considered
[c] Number of euthanized animals showing decubitus or nervous signs for more than 12 h
[d] Bacteriological analyses following post-mortem examination; presence of *S. suis* in at least 1 organ or in blood

to study the antibody response in the natural host.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

Two *S. suis* capsular type 2 strains were used in this study. Strains 999 and 1330 were isolated from pig tissues in the laboratory of clinical bacteriology of the Faculty of Veterinary Medicine, University of Montreal. They were identified as *S. suis* capsular type 2 using a procedure already described (2). These strains had previously been tested with an experimental mouse model of infection and in pigs (12,15) and their virulence estimated as follows: 999 highly virulent and 1330 avirulent. For each strain, 3–4 colonies from a 24 h culture on blood agar plates (5% bovine blood) were inoculated in Todd-Hewitt broth (THB) (Difco Laboratories, Detroit, Michigan, USA) and grown overnight. From this broth, 500 µL were added to 50 mL of fresh medium and grown without agitation at 37°C with 5% CO_2 until the desired absorbance (540 nm), or number of bacteria, was reached.

IMMUNIZATION OF PIGS

Upon their arrival, all pigs were tested serologically for the presence of antibodies against *S. suis* serotype 2 antigens. Using an ELISA assay, piglets with low levels of antibodies were kept for the study. Fifteen 4 week-old crossbred piglets were alloted to each of 3 separate, but identically designed experiments (Table I). In each experiment, pigs were divided into 3 groups of 5 animals and immunizations were carried out via the intra-muscular route. In group 1, 5 piglets were injected 3 times with 10^9 colony forming unit (CFU) of strain 1330 (days 0, 7 and 14). In group 2, 5 other piglets were injected 2 times with the same bacterial concentration (days 0 and 14). In group 3, 5 control animals were injected 3 times with sterile THB (days 0, 7, 14). Between days 0 and 21, pigs were examined twice daily to detect clinical signs of infection. Seven days before the first immunization and 7 d after the last immunization, 2 mL of blood were collected in order to evaluate the antibody response to *S. suis* capsular type 2 cellular proteins by ELISA and to some specific *S. suis* proteins by Western blot. Guidelines from the Guide to the Care and Use of Experimental Animals from the Canadian Council on Animal Care were followed during the experiment.

EXPERIMENTAL INFECTION AND ASSESSMENT OF CLINICAL SIGNS

Pigs were challenged on day 21 by intravenous injection of 10^8 CFU of strain 999 as described by Quessy et al (13). Experimental infection was carried out for a period of 10 d and clinical signs were monitored twice daily for 1 h. Recorded clinical signs included lameness, persistent lateral or ventral decubitus, fever (> 40.5°C), as well as nervous signs such as incoordination, paddling, and opisthotonos. Pigs in decubitus or manifesting nervous signs for more than 12 h, were euthanized for ethical reasons. A necropsy was performed on these animals and tissues such as blood, lung, liver, spleen and brain were cultured for the presence of *S. suis* according to a procedure already described (2). Remaining pigs were euthanized after the 10 d period and submitted for necropsy, where special attention was paid to the immunization site in order to detect any lesions caused by intramuscular injection of the vaccinal strain.

SDS-PAGE

Twenty-five milliliters (mL) of an overnight culture of the strain 999 were centrifuged (12 500 x *g*) for 20 min and resuspended in 1 mL of K_2HPO_4 (0.1 M, pH 7.0), processed in a French Press (16) (SLM, Amico, Urbana, Illinois, USA) (Mini-cell, 20 000 PSI, 3 times), treated with lysozyme (5 mg mL^{-1}) for 4 h at 37°C and centrifuged again (12 500 x *g*) for 20 min at 4°C. Cellular proteins present in the supernatant were harvested, mixed with equal volume of solubilization buffer, boiled 4 min and processed in 10% polyacrylamide vertical slab gels (with 4.5% stacking gels) (17). Gels were stained with Coomassie blue or transferred to nitrocellulose.

WESTERN BLOT ANALYSES

Following SDS-PAGE, material was transferred to the nitrocellulose membrane by electroblotting in a transblot apparatus (Bio-Rad, San Francisco, C) with methanol-Tris-glycine buffer for 1 h at 100 volts (18). Non-reacting sites on nitrocellulose membrane were blocked for 1 h with casein 2% (w/v). The membrane was incubated for 1 h with 1:200 (v/v) dilutions of each pig serum before and after vaccination. After washing, the

membrane was incubated for 1 h with a peroxidase conjugated goat anti-pig IgG (Jackson Immuno Research,West Grove, Pennsylvania, USA). After washing, the presence of bound antigens was visualized by reacting the nitrocellulose membrane with 0.06% 4-*chloro*-1-naphtol (Sigma) in cold methanol mixed to 0.02% H_2O_2 in Tris-NaCl. Apparent molecular weights were calculated by comparison with standards of known molecular weight (Bio-Rad).

SEROLOGICAL RESPONSE OF PIGS FOLLOWING VACCINATION

Porcine sera were tested using an ELISA procedure. Flat-bottom polystyrene microtiter plates (NUNC Immunoplates, Copenhagen, Denmark) were coated at 4°C for 18 h with 0.4 μg of protein extract (strain 1330) in 100 mL of 10 mM phosphate buffer, pH 7.4 (PBS) per well. Then, 100 mL of PBS containing 0.3% (w/v) of casein and 0.005% (v/v) of Tween 20 (Sigma) were added and left 1 h at room temperature in order to block free sites; plates were then washed 3 times. Pig sera were diluted serially in PBS, added in 100 μL amounts to appropriate wells and incubated for 1 h at room temperature. Serum from an axenic pig was used as negative control. The positive control was an anti-strain 999 hyperimmune pig serum obtained from a pig after 6 consecutive immunizations with inactivated bacteria. Well contents were discarded and the plates washed. A volume of 100 μL of goat anti-pig IgG conjugated to horseradish peroxidase (Jackson Immuno Research) diluted 1:2000 in PBS was added to each well and left for 1 h at room temperature. The plates were washed and 100 μL of 0.4 mM 2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma) dissolved in 0.05 M citrate buffer (pH 4.0) with 0.5 M H_2O_2 were added to each well. The absorbance was measured after 30 min of incubation at 22–23°C. Optical density was corrected by substraction of background binding in control wells (coated with PBS). ELISA titers were estimated as the highest dilution that gave an increase in light absorbance at 414 nm (A_{414}) more than twice the mean of the corresponding blank values (without antibody but with conjugate and substrate). Increase in antibody titer was calculated using this formula: titer obtained on day 21 divided by titer obtained on day 0. The mean of titer increase was calculated using this formula: total titer increase for each animal in the same group divided by the total number of pigs in this group.

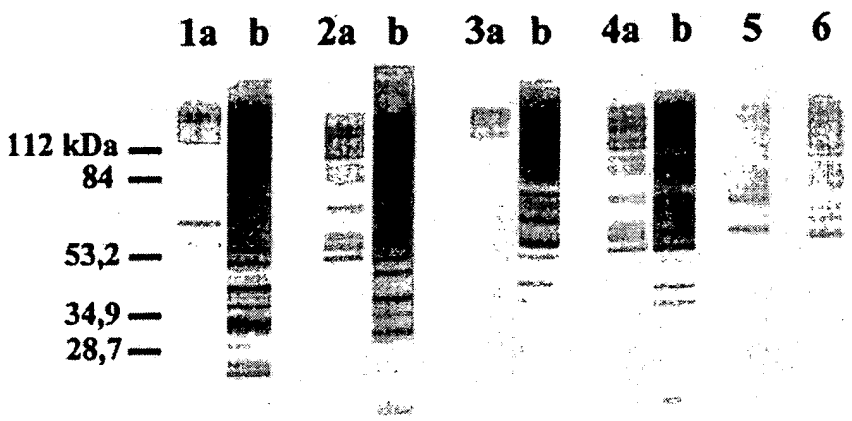

Figure 1. Comparison of the IgG response of pigs to *Streptococcus suis* proteins before (a) and after (b) immunization with the live avirulent strain 1330 by Western blot analysis. Piglets were immunized 3 times (lanes 1 and 2) and 2 times (lanes 3 and 4). Sera from control animals after they received 3 immunizations with sterile Todd-Hewitt broth (lanes 5 and 6). Results using antisera from animals selected from experiment 1 are shown; data were similar in experiments 2 and 3. Western blots were performed after transfer from a 12.5% polyacrylamide gel loaded with virulent strain 999 proteins.

RESULTS

IMMUNIZATION AND SEROLOGICAL RESPONSE OF PIGS

None of the pigs showed lameness, decubitus or nervous signs following immunization with the vaccinal strain of *S. suis*. Necropsy did not reveal any detectable lesions at the immunization site. In the first experiment, a 5.4 fold mean elevation in antibody titer against *S. suis* antigens was observed in pigs that received 3 doses of vaccine (Table I). Antibody titers between 40 000 and 80 000 were observed in these animals, while in animals vaccinated twice, antibody titers varied between 10 000 and 40 000, which corresponds to a 3.5 fold increase. In the control group, titers ranged from 5 000 to 10 000. A similar increase in the mean titer was observed in the 2 other experiments.

WESTERN BLOT ANALYSES

Western blot analyses carried out on sera from immunized pigs, before and after vaccination, showed an apparent increase in the IgG response against the virulent strain 999 cellular proteins (Figure 1 for experiment 1). An IgG response was observed against high molecular weight proteins and against proteins of approximately 40 and 70 kDa (Figure 1). Western blot analyses performed on sera in experiments 2 and 3 gave similar results (data not shown).

EXPERIMENTAL INFECTION

In the 3 experiments, when challenged by an intravenous injection of virulent strain 999, 1 out of the 15 pigs vaccinated 3 times and 3 out of the 15 pigs vaccinated twice showed clinical signs compatible with *S. suis* infection. In contrast, 11 out of the 15 control pigs manifested lameness, decubitus or nervous signs. Finally, 1 out of the 30 immunized pigs and 9 out of the 15 control pigs died (Table I). Lesions attributed to *S. suis* infection were observed and the microorganism was recovered from at least one organ or in blood from all animals having shown decubitus and/or nervous signs. Pathological lesions, and isolation of *S. suis* were recorded in only one of the other pigs (Table I).

DISCUSSION

Previous attempts to protect pigs against *S. suis* type 2 infection, either by injection of formalin killed bacteria (6,7,19) or purified capsular

material (8), have given equivocal results. However, Holt et al (9) using live cultures of *S. suis* induced a protective response in pigs after 8 consecutive immunizations. On the other hand, Quessy et al (14) succeeded in protecting mice against *S. suis* serotype 2 infection after only 2 injections with the live avirulent strain 1330. This strain, when incubated in intra-peritoneal chambers in rats, did not show any increase in the thickness of its capsular material (20). In contrast, when grown in similar conditions, an increase in the thickness of the capsular material was noted for virulent strains and was accompanied by a better resistance to killing by porcine polymorphonuclear leukocytes (12). In mice, it was previously demonstrated that immunization with the avirulent strain 1330 led to the production of IgG recognizing many proteins of different virulent strains (14).

A persistent high-level bacteremia usually precedes the onset of bacterial meningitis (21). Thus the ability of a bacteria to induce and maintain a bacteremia is a major determinant of pathogenicity (22). In this kind of infection, humoral immunity plays an important role. Moreover, the importance of humoral immunity in the pathogenesis of *S. suis* infection was confirmed by Holt et al who found that the protective response was serum-mediated and associated with both IgM and IgG (23). Presence of antibodies and particularly IgG at the bacterial surface could increase recognition and then stimulate uptake by phagocytes (24). However, the outcome of interactions between bacteria and phagocytes is important in determining the level of bacteremia and the incidence of meningitis (25). Since replication of virulent strains of *S. suis* within murine macrophages was shown by Williams (22), cellular immunity could also be determinant. Thus, in a live vaccine, organisms act as endogenous antigens and tend to trigger a response dominated by cytotoxic T-cells (26). In contrast, inactivated organisms, act as exogenous antigens and stimulate a response dominated by helper T-cells (26). If *S. suis* can survive inside phagocytes, both types of immunity would be required to eliminate all bacteria. Since activation is important in the control of organisms inside phagocytes, the control of *S. suis* infections would be improved with T-cell-mediated immune response, along with antibodies. Live bacteria are much more capable of activating phagocytes than inactivated organisms (26). The fact that a 1.8 fold increase in mean titer protected animals in the 3rd part of our assays would indicate that cellular immunity would have contributed, in a large proportion, to the protection observed.

In conclusion, protection against *S. suis* type 2 infection was observed in pigs after 2 or 3 vaccinations with the live avirulent strain 1330. This protection seems to be related to the presence of antibodies against some of *S. suis* cellular proteins and to cellular immunity. However, more studies are needed to assess the use of a live strain of *S. suis* in a vaccine for pigs.

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APPENDIX C
Related Proceedings Appendix (37 C.F.R. §41.37(c)(1)(x))

none